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STUDIES ON IMP DEHYDROGENASE AND GMP REDUCTASE

by

LARRY BROX ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "STUDIES ON IMP DEHYDROGENASE AND GMP REDUCTASE", submitted by Larry Brox in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

The enzymes involved in the formation of AMP or GMP from IMP, and those which catalyze the reverse reactions have not been extensively studied. As a result, a study has been undertaken on two of these enzymes, namely, IMP dehydrogenase and GMP reductase. The source of these enzymes was the P-14 mutant of *Aerobacter aerogenes*, strain 1033. This mutant lacks the XMP aminase enzyme which converts XMP to GMP. As a result, there is a derepression on the synthesis of IMP dehydrogenase which is the enzyme catalyzing the prior IMP to XMP transition. This mutant is then a good source for the start of the purification of this enzyme.

By making certain modifications in the method used in this laboratory for the purification of IMP dehydrogenase, it has been possible to obtain preparations of this enzyme which are almost completely homogenous with respect to protein exhibiting IMP dehydrogenase activity. The purification procedure involves the sonication of bacteria, the removal of nucleic acids with protamine sulfate, an ammonium sulfate fractionation, column chromatography on phosphocellulose, and in certain cases column chromatography on hydroxyapatite. The GMP reductase was partially purified by the same process.

Polyacrylamide electrophoresis, sucrose-gradient centrifugation, and ultracentrifugation experiments indicate the purified IMP dehydrogenase preparations contain two major protein species with one or two minor components. Four activity bands corresponding to the four

protein bands are evident in the disc electrophoresis experiments. With a 0.2% protein solution, values of 9.3 S and 13.1 S were obtained for the sedimentation coefficients of the two sucrose-gradient peaks, and 9.1 S, 12.1 S, and 15.1 S for the three Schlieren peaks seen in the ultracentrifuge. If 0.1 M KCl was not included in the sucrose-gradient centrifugations, values of 19.2 S and 27.2 S resulted which indicated ionic strength may have an effect on the degree of aggregation. Molecular weights of about 200,000 and 300,000 for the centrifugation protein species were estimated from the $s_{20,w}$ values. 3 M urea resulted in a single 6.4 S peak in the ultracentrifuge and gave rise to one electrophoretic band in the polyacrylamide gels.

The initial velocity and product inhibition data on IMP dehydrogenase suggests this system may be described by an ordered Bi Bi kinetic model. In this system, one is faced with the problem of whether or not to treat the water molecule, which is presumably the ultimate source of the C-2 oxygen of XMP, as an actual substrate. At pH 8.1, values of 2.0×10^{-5} M and 1.1×10^{-3} M are obtained for the Michaelis constants of IMP and NAD, respectively. The dissociation constants for IMP, XMP, and GMP which is a competitive inhibitor of IMP are 2.0×10^{-5} M, 1.2×10^{-4} M, and 1.3×10^{-4}, respectively. The forementioned values of the kinetic parameters were calculated using the ordered Bi Bi kinetic model. At pH 6.0, the same kinetic model appears applicable, but the maximum velocity has decreased some 30-fold, and the dissociation constant of IMP increased ten-fold.

Inosine 5'-phosphorothioate, 5'-thio-IMP, and 5'-amino-IMP are IMP phosphate analogues and are substrates for the IMP dehydrogenase. Where-

as the maximum velocities, relative to IMP, for these analogues vary only about 30%, the dissociation constants vary some 40-fold. These results substantiate the contention that the phosphate moiety is specifically involved in the binding of IMP.

It has been reported that 6-chloropurine nucleotide progressively inactivates IMP dehydrogenase, and that this inactivation is retarded by IMP. Evidence is presented that this inhibitor reacts covalently with an enzymic sulfydryl group to produce the inactive enzyme. A study of the rate of this inactivation as a function of 6-chloropurine nucleotide concentration indicates that the enzyme forms a reversible complex with the enzyme prior to the actual inactivation. From this study, a value of 2.6×10^{-4} M was obtained for the dissociation constant of the inhibitor and 0.125 $\sec^{-1}$ for the first-order rate constant of the actual inactivation step. The 6-chloropurine nucleotide used in this study was synthesized by a new method.

The partially purified GMP reductase was progressively inactivated by 6-chloropurine nucleotide, 2-amino-6-mercaptopurine nucleotide, and 6-mercaptopurine nucleotide. GMP offered protection from such inactivation, and glutathione could prevent and reverse the inhibition caused by the latter two nucleotides. Sulfydryl reagents such as p-mercuribenzoate (PMB), iodoacetate, and iodoacetamide also inhibited the GMP reductase. GMP could protect this enzyme from the iodoacetates but not from PMB. NADPH had no apparent protective effect. Glutathione reversed the PMB inhibition completely if added to the inhibited system within 30 minutes. These results suggested an essential sulfydryl group may be in the region of the GMP binding site, and hence, the three synthetic nucleotide in-

activators may be first specifically binding in this site and then participating in a covalent reaction with some neighboring and reactive enzymic group. It was also found that ATP was a potent inhibitor of the GMP reductase. Whereas GMP could completely reverse the ATP inhibition, ATP did not offer protection as did GMP against the inactivation by 6-chloropurine nucleotide.

The subsequent kinetic study on GMP reductase suggested a random Bi Ter kinetic model for this enzyme. At pH 7.5, the Michaelis constants for GMP and NADPH were 7.2×10^{-6} M and 1.3×10^{-5} M, respectively. The dissociation constants for GMP and NADPH with the free enzyme were 8.8×10^{-5} M and 1.65×10^{-4} M. It was also found that magnesium chloride at 1×10^{-3} M caused a 40% increase in the maximum velocity of GMP reductase. The magnesium had no significant effect on the Michaelis constants for GMP and NADPH, but decreased the corresponding dissociation constants 3.5-fold. This magnesium effect is not at present understood.

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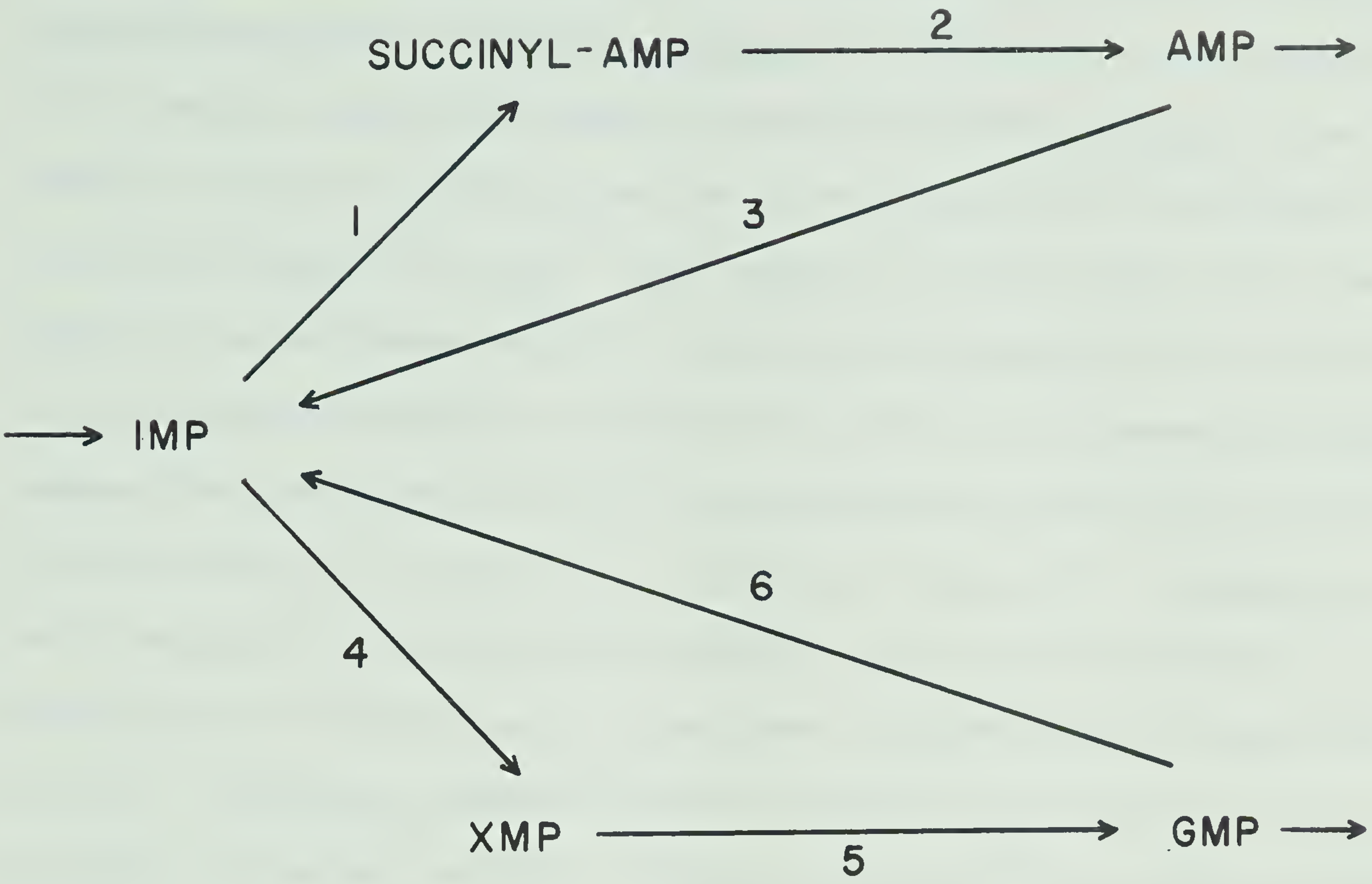
I. INTRODUCTION

The wide involvement of purine nucleotides in such phases of cellular metabolism as nucleic acid synthesis, protein synthesis, energy transfer, etc., no longer requires documentation. Most cells can synthesize their own purine nucleotides by the so-called purine de novo pathway (1), and most can also utilize preformed purines, such as the free bases (2, 3), or perhaps certain nucleosides (4) when these may be available.

The series of essentially irreversible reactions illustrated in Figure 1 have been shown to provide a means by which the purine nucleoside monophosphates may be interconverted (5, 6, 7, 8, 13, 21). IMP may be considered the end or branch-point of the de novo pathway. Once AMP and GMP are formed, they may be further phosphorylated to the more biologically active di- and triphosphate levels. The monophosphates are also subject to catabolic processes which usually involve an initial dephosphorylation to the nucleoside level. This dephosphorylation may be effected by the nonspecific acid and alkaline phosphatases, or by the specific 5'-nucleotidases present in various cells (9).

While the existence of the enzymes shown in Figure 1 has been demonstrated from a variety of sources, they have not, with a possible exception of one or two, been extensively studied from a kinetic or physical chemical point of view. This thesis will describe additional studies on two of these enzymes, namely, IMP dehydrogenase and GMP reductase.



Reaction	Enzyme
1	Adenylosuccinate synthetase or IMP:L-aspartate ligase (GDP) (6.3.4.4)
2	Adenylosuccinate lyase or Adenylosuccinate AMP-lyase (4.3.2.2)
3	AMP deaminase or AMP aminohydrolase (3.5.4.6)
4	IMP dehydrogenase or IMP:NAD oxidoreductase (1.2.1.14)
5	XMP aminase or XMP:ammonia ligase (AMP) (6.3.4.1) or XMP:L-glutamine amido-ligase (AMP) (6.3.5.2)
6	GMP reductase or NADPH:GMP oxidoreductase (deaminating) (1.6.6.a)

Figure 1: Enzymatic steps involved in purine mononucleotide interconversions.

During the period of 1954 to 1956, Magasanik and coworkers isolated a guanineless mutant of *Aerobacter aerogenes*. This mutant lacked the ability to produce nucleic acid guanine either by synthesis *de novo* or from adenine, and was observed to give rise to an accumulation of xanthosine in the culture fluid (10, 11, 12). This suggested to these workers that some derivative of xanthosine may be an obligatory intermediate in the formation of the nucleic acid guanine, presumably via IMP. In 1957 Magasanik *et al*. reported the isolation and partial purification of an enzyme from various wild type and mutant strains of *A. aerogenes*, *E. coli* and *S. typhimurium* which oxidized IMP to XMP with NAD serving as the electron acceptor (13). Hypoxanthine or inosine were not substrates, and NADP would not substitute for NAD. Potassium or ammonium ions were required, and a thiol was also necessary for maximal activity. Values of 1.39×10^{-5} M and 3.80×10^{-3} M were reported as the Michaelis constants, at pH 7.8, for IMP and NAD, respectively. These values are only apparent values because a complete initial velocity study utilizing various fixed substrate concentrations was not carried out. The reaction was essentially irreversible in the *in vitro* assays. Similarly, the intact guanineless mutant was unable to convert ^{14}C-labeled xanthine to nucleic acid adenine (13).

About the same time as the above studies were in progress, two other laboratories demonstrated the presence of a DPN-linked IMP dehydrogenase in cell extracts of rat bone marrow (14) and pigeon liver (15). Since these initial demonstrations of IMP dehydrogenase activity, this enzyme has been subsequently found in pea seeds (16), in L-cells (17), in Ehrlich cells (17, 18) and in Sarcoma 180 cells (19). The IMP dehydrogenases examined from these sources all required a monovalent cation, of which potassium was the most effective. The enzyme from pea seeds, Sarcoma 180, and Ehrlich cells

was not stimulated by thiols; but the enzymes from the first two of these sources were susceptible to sulfydryl reagents such as $HgCl_2$, p-mercuribenzoate, the iodoacetates, and N-ethylmaleimide. IMP, but not DPN, protected the Sarcoma 180 enzyme from N-ethylmaleimide and iodoacetate but not from the organic mercurials (19). The IMP dehydrogenases are quite sensitive to dialysis, but in one case (18) almost full activity was recovered by adding back a small amount of the dialysate to the dialyzed enzyme. This observation was not pursued but EDTA was not inhibitory in this system.

The only complete initial velocity study of IMP dehydrogenase so far reported was that of Hampton and Nomura on the enzyme from the P-14 mutant of *A. aerogenes* (20). These workers reported Michaelis constants of 2.1×10^{-5} M and 1.1×10^{-3} M, at pH 8.1, for IMP and NAD, respectively. Previously reported constants for IMP ranged from 1.4 to 4.0×10^{-5} M, and from 0.25 to 3.8×10^{-3} M for NAD. The Michaelis constant for NAD with the Sarcoma 180 enzyme was however much smaller at 8×10^{-5} M (19).

From an initial velocity study of any given enzyme system one obtains the Michaelis constants for the substrates involved. These are operational constants in that they represent a concentration for half maximal velocity when all other substrates are saturating. For the more complicated two and three substrate reactions, they do not necessarily represent the binding process alone (40). The kinetic analysis of IMP dehydrogenase was extended in this present work by product inhibition studies so that a working kinetic model for this enzyme might be selected. Such a model would describe the order in which substrates added to and were released from the enzyme. Once a particular model is selected, more detailed information such as actual dissociation constants may be real-

ized from the kinetic data (40).

Various natural purine nucleotides have been reported to inhibit IMP dehydrogenase, and this phenomenon has been suggested as perhaps involved in the control of purine nucleotide interconversions (21). The bacterial enzyme was inhibited in a competitive manner with respect to IMP by GMP and GDP, whereas GTP and ATP gave little inhibition (21, 20). A K_i value of 1.3×10^{-4} M has been reported for GMP (20). The mammalian Sarcoma 180 enzyme is inhibited by XMP, AMP, and GMP (19). The GMP was competitive with IMP with a K_i of 3×10^{-4} M. Once a kinetic model for IMP dehydrogenase is selected, it will be possible to determine the dissociation constant for GMP. From the relative values of the dissociation constants for IMP, XMP, and GMP a better idea may be obtained as to the possibility of either XMP or GMP exerting any *in vivo* control on IMP dehydrogenase.

The similarity of the oxidation at the C-2 position of the purine ring catalyzed by IMP dehydrogenase and by the xanthine oxidases, particularly the chicken liver enzyme which also uses NAD as the electron acceptor, prompted Powell *et al*. to compare the properties of these enzymes (22). They found these enzymes to be very dissimilar. The xanthine oxidases contain protein-bound flavin and non-heme iron while IMP dehydrogenase does not; the oxidases may transfer electrons directly to a variety of organic dyes while IMP dehydrogenase can not; and typical xanthine oxidase inhibitors such as cyanide and methanol are without effect on the bacterial IMP dehydrogenase which they used.

IMP dehydrogenase from bacterial (23, 20) and mammalian sources (24) can be inhibited in a progressive manner by 6-chloropurine, 6-mercaptopurine and 2-amino-6-mercaptopurine nucleotides but not by the corre-

sponding nucleosides. The progressive nature of these inactivations was taken as evidence that these inhibitors reacted covalently with IMP dehydrogenase to give an inactive enzyme (20, 23). These inactivations are of interest due to the reported chemotherapeutic effects of the free base form of the forementioned inhibitors on various mammalian tumors (25, 26, 27). The observation that IMP protected the IMP dehydrogenase from the inactivating effect of these synthetic nucleotides (20, 24) suggested these reagents may be reacting at the binding site of IMP, and hence, may be examples of the "site-specific" concept which has been elaborated by Baker (28).

Baker (28a) postulated in 1959 that it may be possible to specifically inactivate a given enzyme by the use of modified substrate analogues containing some reactive chemical group. When this modified analogue binds to the enzyme, the newly introduced group may be able to react covalently with a nearby enzymatic group which was either part of or close to the substrate binding site, and thus partially or completely inactivate the enzyme. He has further proposed that by utilizing an enzyme-substrate complex in this manner much higher rates of inactivation may be obtained with very low inhibitor concentration as compared to the unspecific, one-step, bimolecular reactions of general alkylating reagents. A classical example of an inhibitor which first forms an enzyme complex and then reacts chemically to cause inactivation is that of 0,0-dimethyl-S-(1,2-dicarbomethoxyethyl)-phosphorothiolate with cholinesterase (29).

Part of this present study will deal with the examination of the rate of the inactivation of IMP dehydrogenase by 6-chloropurine nucleotide. Such a study was expected to demonstrate if this inhibitor did form a complex with IMP dehydrogenase prior to inactivation, and if so, perhaps

some idea might be obtained as to the value of the dissociation constant. The nature of the enzymic group reacting with the inhibitor is of interest since such a reactive group may be involved in binding or catalysis.

It has become apparent that many of the naturally occurring and synthetic purine derivatives which have been observed to have antineoplastic action exert this action, at least in part, in the form of the nucleoside 5'-phosphates (30). Since the phosphate group is necessary for activity, it is of interest to study what contributions this group makes to enzyme binding. Furthermore, as phosphate monoesters of various types are very common in biological material it would be of interest to see if these contributions are at all general for different monoesters. In this laboratory the role played by the phosphate moiety of a nucleotide in enzyme binding has been studied by examining the substrate and inhibitory properties of various synthetic phosphate analogues of IMP with such enzymes as IMP dehydrogenase and adenylosuccinate synthetase (31). This report contains the results of experiments with some newly synthesized analogues (41) and IMP dehydrogenase. It is hoped that any general knowledge regarding nucleotide binding will be useful in deciding the mode of action of various nucleotide antineoplastic agents now in use and in the future design of better agents.

In the early 1950's it had been reported that _E_. _coli_, _A_. _aerogenes_ and various other related bacteria were capable of using either adenine or guanine present in the medium for the synthesis of both nucleic acid guanine and adenine (32, 33, 34). Since the IMP dehydrogenase catalyzes an essentially irreversible reaction, it became obvious that the conversion of exogenous guanine to adenine nucleotides was not proceeding in a reverse manner along the IMP to XMP to GMP pathway. However, in 1960 Mager

and Magasanik succeeded in isolating and partially purifying an enzyme from *E. coli*, *A. aerogenes* and *S. typhimurium* which reductively deaminated GMP to IMP with NADPH as the electron donor. This enzyme, which they named GMP reductase, would not deaminate guanine or guanosine, and NADH could not substitute for NADPH. The reverse reaction from IMP to GMP could not be detected. This reaction was inhibited strongly by ATP, but this could be almost completely reversed by increasing the GMP concentration.

Information about this reductase activity in higher organisms is still very limited. Abrams and Goldinger demonstrated an increased production of hypoxanthine when guanine was incubated with rabbit bone-marrow slices (35). It has also been demonstrated that a small amount of radioactivity can be detected in the adenine or nucleic acid when ^{14}C-guanine is injected into rats (36). Rat-liver extracts can convert some guanine to hypoxanthine. This conversion is believed by one group (37) to be effected at the nucleotide level as in bacteria, but in similar experiments another group concluded this conversion occurred at the base level via guanine deaminase and xanthine oxidase (38). Mager *et al*. have more recently shown that in human and rabbit red blood cells there is a NADPH-linked reductive deamination of GMP (39).

During purification procedures for the IMP dehydrogenase from *A. aerogenes*, it was found in this laboratory that a partial purification of GMP reductase was also effected (84). Initial velocity and product inhibition experiments will be described in Chapter VIII of this thesis which elucidate a possible kinetic model for this enzyme. The 6-chloropurine, 6-mercaptopurine and 2-amino-6-mercaptopurine nucleoside 5'-phosphates have also been tested in this system to see if these poten-

tial inactivators produce inhibitions of the type seen in the IMP dehydrogenase system (23). Another part of this report describes the effect of some common sulfydryl reagents on the GMP reductase.

II. ENZYME SOURCE AND PURIFICATION

1. Source

The source of both the GMP reductase and the IMP dehydrogenase used in these studies was a mutant of Aerobacter aerogenes. This P-14 mutant was selected by UV irradiation of Aerobacter aerogenes, strain 1033 (42). Magasanik reported that a guanine source such as guanine itself, guanosine, GMP, or 2,6-diaminopurine was required to support the growth of this organism (42). It was further established that this mutant lacks xanthosine-5'-phosphate:ammonia ligase (AMP) which is the enzyme that converts XMP to GMP in bacteria. As a result, there is a derepression of the synthesis of IMP dehydrogenase which is the enzyme catalyzing the step immediately prior to this amination of XMP to GMP. The amount of the IMP dehydrogenase synthesized was a function of the guanine concentration in the medium. The lowest concentration of guanine which would give a suitable growth rate resulted in the largest synthesis of IMP dehydrogenase. Under suitable conditions this P-14 mutant contained 28 times more IMP dehydrogenase than the parent strain 1033 (13). This mutant is obviously a good source for the purification of IMP dehydrogenase since it provides an initial 28-fold purification factor.

The P-14 strain of Aerobacter aerogenes was grown commercially by the Miles Chemical Co. of Elkhart, Indiana, in a liquid medium consisting of 13.6 gm $Na_2HPO_4 \cdot 12H_2O$, 12.6 gm KH_2PO_4, 0.4 gm $MgSO_4 \cdot 7H_2O$, 0.02 gm $CaCl_2$, 2.0 gm vitamin free Casamino acids and 20 gm glycerol per 1 liter of medium. Preliminary batches indicated that 10 mg of guanine per liter of medium gave the best yield of IMP dehydrogenase. Of the two large batches

of bacteria ordered, however, the latter contained approximately one-fifth the enzyme activity on a protein basis. The cultures were incubated for 19 hours at 34° when the bacteria were collected by centrifugation and stored as a frozen paste.

2. IMP Dehydrogenase Purification

a. Assay

The spectrophotometric assay employed was based on the increase in optical density at 340 mμ due to NAD oxidation or at 290 mμ due to the IMP to XMP transition, and was followed continuously at 23-24° on a Cary model 15 spectrophotometer. Assay mixtures contained 24 μmoles of Tris-citrate buffer, pH 8.1, 2 μmoles of glutathione, 100 μmoles of KCl, 2 μmoles of NAD and 0.4 μmoles of IMP in a final volume of 1.0 ml. IMP was obtained from P-L Biochemicals; NAD from Schwarz; and glutathione, XMP, and NADH from Calbiochem. Other chemicals were of reagent grade and obtained from various commercial suppliers. Stock solutions of NAD and glutathione were neutralized with KOH prior to their use. Assays were started by addition of IMP to the 1 cm light-path, sample cuvet. The reference cuvet lacked only IMP. An arbitrary unit of enzyme activity was defined as that which effected an increase in optical density of 1.0 in 1 minute. The protein concentration was determined by the Lowry method (48) using bovine serum albumin as a standard.

Enzyme activity was a linear function of protein concentration over at least a five fold range on either side of that amount used in any type of experiment. Assays were linear with time during approximately the initial 50% of the reaction. If followed to completion, the total optical density change would correspond, within experimental error, to complete

conversion to product of whichever substrate was not in excess. This indicates that the equilibrium position is shifted very far to the side favoring XMP formation.

b. Phosphocellulose Column Chromatography

An initial purification procedure for the IMP dehydrogenase from *Aerobacter aerogenes* has been reported from this laboratory (20). Very briefly, it involved disrupting the bacteria by sonication (step 1) and subsequently removing nucleic acids with protamine sulfate (step 2). After removal of excess salt, the precipitate of a 45% ammonium sulfate precipitation (step 3) was applied to a phosphocellulose column and subjected to stepwise elution with KCl. The dehydrogenase was eluted with 0.5 M KCl (step 4) and had a specific activity of 4.5 to 5.0 units per mg of protein which corresponded to a 25-30 fold purification over the crude sonicates.

The previously described purification procedure invoked dialysis for the removal of excess $(NH_4)_2SO_4$ prior to phosphocellulose chromatography. This step was responsible for 60 to 70% of the total loss of activity throughout the entire purification process (20). To circumvent this problem, Sephadex (G-25, fine) was used for this desalting step. The Sephadex was equilibrated with 0.02 M potassium phosphate buffer (pH 7.4) and packed in Sephadex columns which were run in a cold room at 4^{o}. Utilizing this method of desalting, practically quantitative amounts of enzyme activity from the ammonium sulfate step 3 could be applied to the phosphocellulose column.

In an attempt to scale up the purification process, experiments with larger columns were undertaken. Whatman phosphocellulose (7.4 mequiv/gm) was subjected to the alkali, acid, and alkali cycling procedure as described by Pederson and Sober (43). The phosphocellulose was used only once

and then discarded. Kontes Chromatoflex columns of 2.5 x 50 and 5.0 x 50 cm were packed on Teflon supporting disks to a height of 40 cm with the washed phosphocullulose. These columns were packed under pressures of 10 and 5 psi, respectively, and then equilibrated in a cold room at 4° with approximately 8 to 10 volumes of 0.02 M potassium phosphate butter at pH 7.4.

To the 2.5 x 40 cm column was applied the desalted step 3 fraction originating from about 60 grams of wet packed bacteria. The phosphocellulose to protein weight-ratio was almost 50% higher than that used in the studies of Hampton and Nomura (20). The flow rate was maintained at 45 mls per hour with a Milton Roy piston pump.

Two protein-activity elution profiles are shown in Figure 2. The majority of the IMP dehydrogenase activity was eluted in either the latter portion or the trailing edge of the 0.25 M KCl peak. This differs from the earlier purification (20) where activity was eluted in the 0.50 M KCl peak. In addition, partial resolution of this protein peak was observed. The increased resolution might be attributed to the more favorable ratio of phosphocellulose to protein, but this would not be expected to alter the salt requirement of elution. The consistency of the protein and activity profiles is evidenced by the fact that 17 or 18 columns, packed from perhaps a dozen washed batches of phosphocellulose, gave results similar to those shown in Figure 2. Two different batches of Whatman phosphocellulose gave the same results. Alternative explanations for the elution of activity with 0.25 M KCl may be that there was some difference in washing and packing procedures for phosphocellulose, or that the use of Sephadex rather than dialysis for desalting may be associated with an altered affinity for the phosphocellulose. This latter explanation is

perhaps favored since one knows from the loss of activity that dialysis is affecting the enzyme in some manner.

The specific activity of the IMP dehydrogenase fractionated on column A of Figure 2 was 31.4 units per mg of protein which corresponded to a purification over crude extracts of 195-fold. The recovery of activity from the phosphocellulose columns ranged from 70 to 85%.

The larger 5.0 x 40 cm columns of phosphocellulose were also employed using the same relative sample size, *i.e.*, the desalted step 3 fraction from approximately 240 grams of wet packed bacteria. Essentially the same protein and activity profiles as shown in Figure 2 were obtained. At the time these columns were used the second commercial batch of bacteria was in use. As mentioned earlier, this batch had been grown from a different culture, and the IMP dehydrogenase content was about one-fifth the first batch, so that even though the larger columns accommodated more protein there was no increase in yield of dehydrogenase per column.

The forementioned purification together with increased IMP dehydrogenase production by this derepressed mutant gives rise to a 5,500-fold purification over the level of IMP dehydrogenase found in the wild type *Aerobacter aerogenes*.

The electrophoretic and centrifugation characteristics of the better phosphocellulose fractions described in the next chapter indicate they are very nearly homogeneous with respect to IMP dehydrogenase. Various stability studies on the bacterial enzyme have been reported from our laboratory (20).

c. Additional Column Chromatography

As seen in Figure 2, the IMP dehydrogenase activity was sometimes eluted well out in the trailing edge of the 0.25 M KCl peak while at other

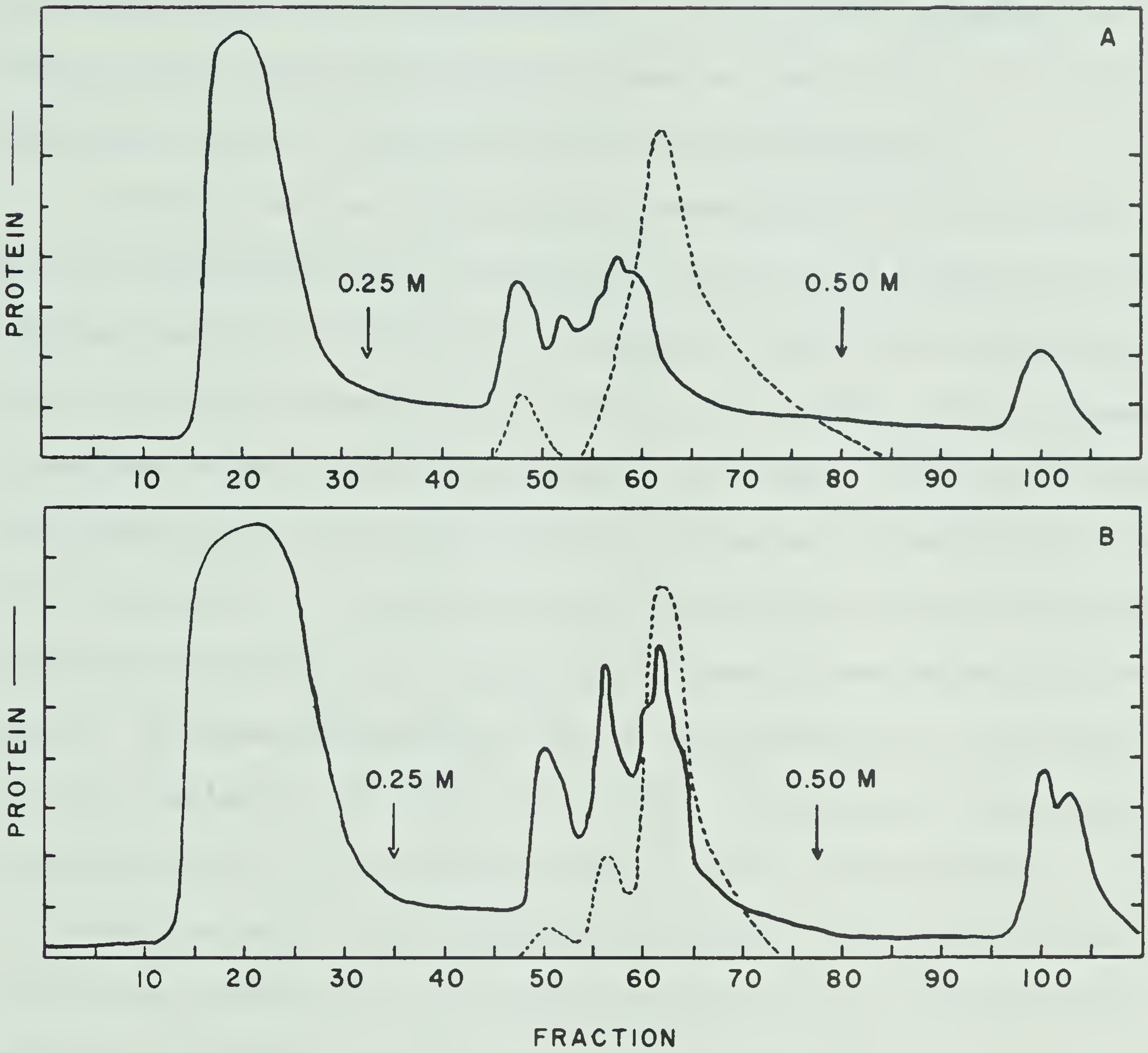


Figure 2: Typical stepwise elution profiles of protein and IMP dehydrogenase activity from phosphocellulose columns. Frame A shows activity eluted in trailing edge of 0.25 M KCl peak and Frame B shows activity eluted slighty earlier.

times it appeared slightly earlier. The former preparations were essentially homogeneous with respect to IMP dehydrogenase activity as evidenced by polyacrylamide disc electrophoresis. It was however necessary to further purify preparations, such as shown in Figure 2-B, in which dehydrogenase activity eluted earlier in the 0.25 M KCl peak.

It was found that hydroxyapatite chromatography was a suitable means of further purifying the phosphocellulose enzyme preparations when this was required. Calobiochem hydroxyapatite powder was suspended in 0.001 M potassium phosphate buffer at pH 7.4, decanted, and resuspended in the same buffer. This was repeated 8 to 10 times. It was found that centrifuging these suspensions instead of allowing the hydroxyapatite to settle by itself, or vigorous stirring of the hydroxyapatite suspensions caused extremely slow flow rates when this material was packed into the column. This would presumably be due to the breaking up of the calcium phosphate aggregates. A thick slurry of the hydroxyapatite suspension was poured into a 2.5 cm Sephadex column to give a packed height of 7 cm. The column was packed under atmospheric pressure. The column was then equilibrated overnight with at least 20 volumes of the 0.001 M starting buffer. All of these operations were carried out at 4^{o}.

Twenty to twenty-five mg of phosphocellulose step 4 protein was precipitated by addition of solid $(NH_4)_2SO_4$ to 65% saturation, resuspended in the 0.001 M potassium phosphate buffer, and applied to the hydroxyapatite column. Figure 3 shows a stepwise elution profile using increasing concentrations of the phosphate buffer. A breakthrough protein peak was subsequently followed by an IMP dehydrogenase activity peak. This breakthrough protein peak was not present if the protein corresponding to the IMP dehydrogenase activity which had eluted well out in the trailing edge

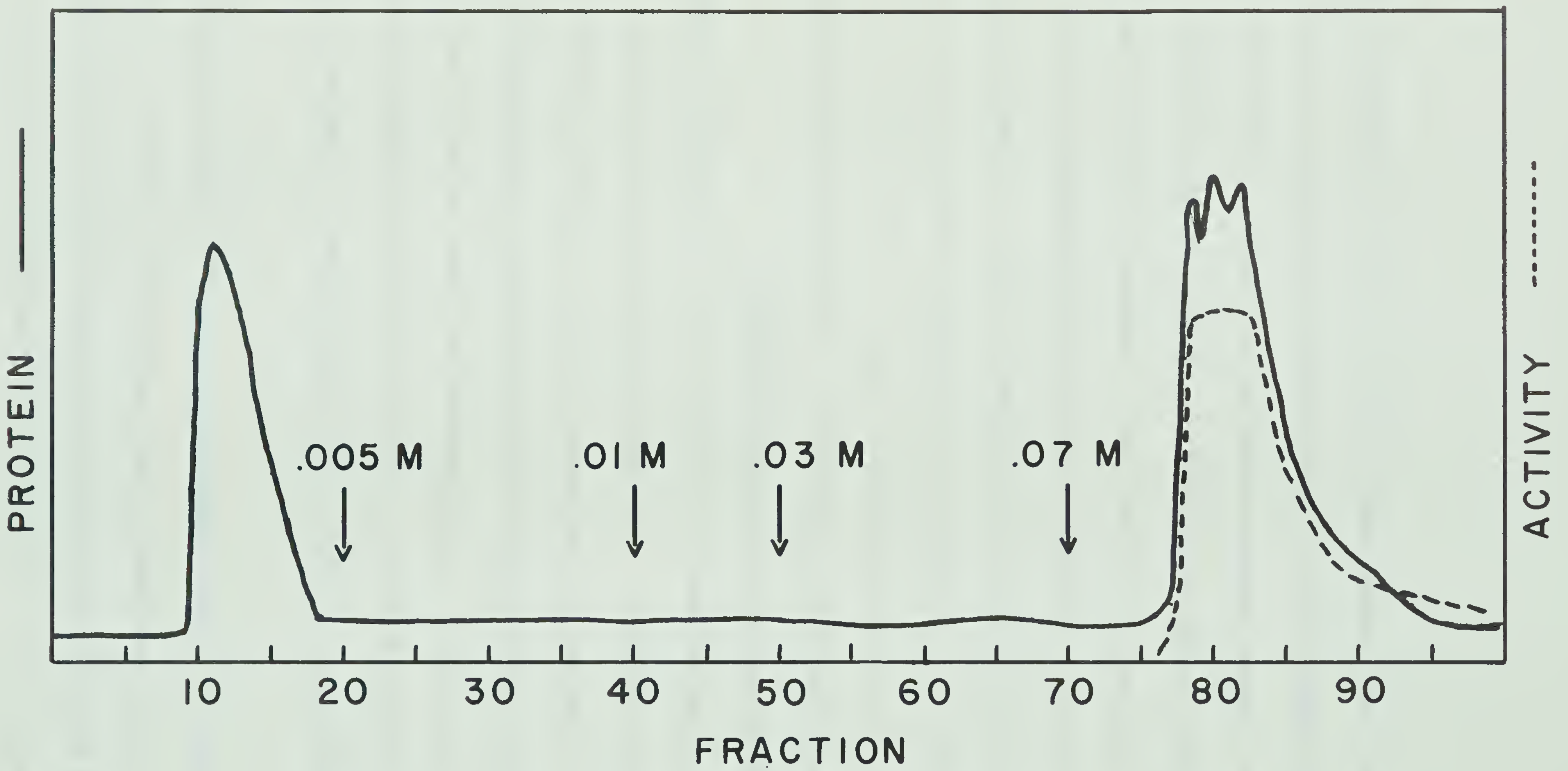


Figure 3: Elution pattern from hydroxyapatite column chromatography. Fractions 58-70 of the phosphocellulose column shown in Figure 2-B were precipitated with ammonium sulfate (see text) and applied in 0.001 M phosphate buffer. The concentration of phosphate buffer was increased stepwise as shown. Six 4-ml fractions were collected per hour.

of the 0.25 M KCl phosphocellulose peak was chromatographed on hydroxyapatite. Specific activities of the active hydroxyapatite preparations varied from 24 to 28 activity units per mg of protein with recovery usually in excess of 80%. The electrophoretic and sedimentation behavior of these preparations was similar to those obtained for the best phosphocellulose fractions.

Column chromatography of step 4 IMP dehydrogenase on Whatman DEAE-cellulose with a linear KCl gradient in 0.02 M potassium phosphate buffer of pH 7.4 was not as successful as hydroxyapatite since activity was much more diluted. Attempts to concentrate these fractions by freeze-drying resulted in a loss of over 50% in enzymatic activity. These fractions could be concentrated with about a 10% loss of activity using Sephadex G-25-C as described in the Pharmacia literature, but this process required large amounts of Sephadex and was very time-consuming.

Preliminary experiments with molecular sieve chromatography using Sephadex G-100 showed the IMP dehydrogenase activity to be eluted at the void volume of the column and suggested the molecular weight to be in excess of 100,000. Later experiments with Sephadex G-150 or G-200 gave broad protein and activity profiles suggesting the IMP dehydrogenase activity existed in forms of different molecular size. Sephadex G-150 or G-200 were not used extensively due to the difficulty in packing columns to obtain reasonable flow rates. These Sephadex results are similar to some subsequently reported by Powell *et al*. During their purification of the *E. coli* IMP dehydrogenase, they found three activity peaks on Sephadex G-200 columns (22).

3. GMP Reductase Purification

a. Assay

The method of assaying GMP reductase was based on that of Mager and Magasanik (21). The decrease in optical density at 340 mμ due to NADPH oxidation or at 285 mμ due to the conversion of GMP to IMP was followed at 23-24° in 1.0 cm cuvets with a Cary Model 15 spectrophotometer or a Gilford spectrophotometer.

The rate of decrease in optical density remained constant for at least 10 minutes. Final concentrations in assay mixtures (1 ml) were: Tris-HCl buffer (pH 7.5) (4×10^{-2} M), NADPH (2×10^{-4} M), GMP (2×10^{-4} M), and GSH (2×10^{-3} M). GMP was obtained from P-L Biochemicals while NADPH and NADP came from Calbiochem. The reaction could be started equally well by addition of GMP and/or NAPDH or enzyme. The control cuvet lacked GMP in order to correct for any contaminating NADPH oxidase activity. One unit of GMP reductase is defined as the amount giving an optical density change of 1.00/min at 340 mμ.

b. Fraction A

Initial experiments with GMP reductase were done on a crude cell extract from which only nucleic acids had been removed. The cells were disrupted sonically by the procedure of Mager and Magasanik (21) except that the operation was carried out in the presence of 1×10^{-3} M β-mercaptoethanol instead of 5×10^{-3} M glutathione. After removal of cell debris by centrifugation at 14,000 rpm for 15 minutes in a Serval RC-2, the cell extract was treated with one-tenth its volume of 5% aqueous streptomycin sulfate and the precipitate removed by centrifugation for 15 minutes at 14,000 rpm. The supernatant, later referred to as Fraction A, was stored at -20°. Dialysis of the cell extract prior to this streptomycin treat-

ment (21) was not carried out since it led to extensive loss of GMP reductase activity.

c. Column Purification

The procedures used in the IMP dehydrogenase purification also resulted in considerable purification of the GMP reductase. Reductase activity was eluted from the phosphocellulose columns with 0.25 M KCl. An example of one reductase preparation is shown in Table I. The recovery and the elution profile were similar at pH 6.5 or 7.4.

As was the case for IMP dehydrogenase, the dialysis of ammonium sulfate fractions prior to the phosphocellulose chromatography resulted in loss of GMP reductase activity. Thiols had no stabilizing effect. Loss of activity did not seem due to removal of metal ions during dialysis since up to 10 mM EDTA had no effect on the undialyzed preparations. In one instance, activity totally disappeared during dialysis of a step 3 fraction containing 15.1 units of activity, but 8.3 units were subsequently eluted from the phosphocellulose column in the expected position.

The final amount of purification of GMP reductase varied from 40 to 300 fold over the crude extracts. This apparently extreme variation is due to the difficulty in accurately assaying the enzyme during the first three steps of purification. There is present in these fractions some GMP-independent NADPH oxidase activity. In crude extracts the decrease in optical density at 340 mμ brought about by this competing activity may be as much as eight to ten times that attributed to GMP reductase. It was impossible to saturate either the GMP reductase or this competing enzyme(s) with NADPH due to the high UV adsorption of this coenzyme. Greater than 95% of this competing NADPH oxidase activity appeared in the breakthrough peak during the phosphocellulose chromatography. No attempt was made to

TABLE I: Purification of GMP Reductase.

Step	Fraction	Vol. (ml)	Total Protein (mg)	Total Act. (Units)	Sp Act. (units/mg of protein x 10^3)	Purification Factor
1	Crude extract[a]	497	2982	[b]		
2	Protamine sulfate	508	2286	10.2	4.5	1.0
3	Ammonium sulfate	35	788	26.3	33.4	7.4
4	Phosphocellulose	84	71	15.2	212	47.5

[a] From 60 g of washed packed cells. [b] No GMP reductase activity was detected in the crude extract.

identify the electron acceptor in this system although it was observed that oxidized GSH would stimulate this GMP-independent oxidase activity.

During the initial studies on the inhibition of GMP reductase by 6-chloropurine ribonucleoside 5'-phosphate, it was observed that the crude GMP reductase preparations contained an enzyme or enzymes capable of hydrolyzing 6-chloropurine nucleoside and 6-chloropurine nucleotide to inosine and IMP, respectively. This dechlorinase activity, which was not adsorbed on the phosphocellulose, was evidenced by a shift in absorption maxima from 264 to 248.5 mμ together with an increase in optical density which corresponded to that expected from the known extinction coefficients of 6-chloropurine nucleoside and inosine and the related nucleotides. The hydrolyses were analyzed by paper chromatography in butanol-acetic acid-water (5:3:2) and isopropyl alcohol-1% ammonium sulfate (2:1). The 6-chloropurine nucleotide was attacked more slowly than its nucleoside; e.g., about 0.5 mg of step 3 protein catalyzed approximately 50% conversion of 0.1 μmole of nucleotide (R_F values 0.36 and 0.65, respectively) to IMP (R_F values of 0.15 and 0.54) within 40 minutes at pH 7.5. In a similar experiment with 0.07 μmole of 6-chloropurine nucleoside, conversion to inosine was complete within 30 minutes as judged by the above spectral shift and by paper chromatography. Adenosine deaminase activity was detected in the same enzyme preparation. The step 4 fraction contained no detectable amounts of NADPH oxidase or dechlorinase activity. It has been reported that adenosine deaminases from calf intestine (44), rat heart (45) and _Aspergillus_ _oryzae_ (46) possess dechlorinase activity.

d. Properties of GMP Reductase

Fraction A which contained 0.9 mM β-mercaptoethanol did not require additional thiol for maximum activity. The step 4 enzyme fraction (Table

I), in contrast to the purified preparation of Mager and Magasanik (21), did not exhibit an absolute requirement for a thiol, although a thiol was required for maximal activity with almost all the step 4 preparations. GSH, cysteine, or β-mercaptoethanol were equally effective at a concentration of 2 x 10^{-3} M. The thiol stimulation of step 4 fractions varied from zero to ten fold. The activity exhibited in the absence of a thiol disappeared after 1 or 2 days at 4°, whereas the maximal activity obtained in the presence of a thiol remained constant over this period with only a 25% loss within 2 weeks at 4°. It appears as if enzyme activity is dependent upon some sulfydryl group or groups remaining in the reduced form, and that these groups may become oxidized during purification or on exposure to the air.

Fraction A and steps 2 to 4 were stable for at least 4 months at -20°. The activity exhibited in the absence of a thiol was also stable at -20° and started to disappear only upon thawing.

In the presence or absence of a thiol, the reaction velocity was proportional to the step 4 protein concentration over the range examined (15-75 μg/ml). Normal assays contained 25 to 50 μg of protein in the 1 ml volume.

III. ELECTROPHORESIS AND SEDIMENTATION OF IMP DEHYDROGENASE

1. Introduction

Cellulose acetate and polyacrylamide disc electrophoresis were used to obtain an indication of the homogeneity of the purified IMP dehydrogenase preparations. The multiple activity bands observed with disc electrophoresis indicated isoenzymes or some type of molecular aggregates were in existence. To attempt to resolve which was the case, sucrose gradient and analytical ultracentrifugation experiments were undertaken. These experiments indicated the presence of different molecular forms but a comprehensive study has not been possible due to the rather limited amounts of highly purified enzyme available. Some salt and urea effects on this multicomponent system are also described in this chapter.

2. Cellulose Acetate Electrophoresis

Initial attempts to examine the electrophoretic behavior of the IMP dehydrogenase preparations were carried out on Gelman Sepraphore III strips (1" x 6-3/4"). The phosphocellulose purified protein, 15 to 50 μg, was applied to the center of a cellulose acetate strip with either a Gelman sample applicator or a number 00 sable brush. Electrophoretic runs of 1 to 2.5 hours were carried out using the Gelman apparatus with voltage gradients of 25-35 volts per inch. Tris-maleate buffer systems (0.05 M) of pH 7.4 and 8.2 were precooled to 4°.

Protein was stained by overnight treatment with 0.02% Nigrosin in 2% acetic acid. Strips were also stained for IMP dehydrogenase activity by placing a second strip of cellulose acetate, which had previously been

soaked in a tetrazolium reagent mixture (107), in direct contact with the electrophoretic strip. Within 15 minutes the dehydrogenase was evidenced by a blue band. Longer exposures to the tetrazolium soaked strip resulted in the appearance of a brownish background which tended to mask the blue band. After staining for protein or IMP dehydrogenase activity, the strips were fixed several hours in 7% aqueous acetic acid.

The tetrazolium reagent mixture was made just prior to use by mixing 3 stock solutions. The first (2 ml) contained 15 mg of NAD, 10 mg of IMP, 3 mg of GSH and 37 mg of KCl. To this was added 3 ml of a solution containing 1 mg per ml of m-nitro blue tetrazolium chloride (Nutritional Biochemicals Corp.) and 0.3 ml of another solution containing 1 mg per ml of phenazine methosulfate (Nutritional Biochemicals Corp.). The mixing and the staining procedures were done in the dark.

With this procedure one protein band, moving toward the anode, was observed at both pH 7.4 and 8.2. The times of the runs were adjusted so that the band would move 2 to 4 cm. This single band stained for IMP dehydrogenase, and the stain was dependent upon the presence of IMP in the tetrazolium reagent mixture.

3. Polyacrylamide Gel Electrophoresis

A more discriminating examination of the purified enzyme preparations was possible by utilizing a discontinuous pH gradient in polyacrylamide gels as described by Ornstein and Davis (47). A Canalco Model 66 electrophoresis apparatus together with the gel formations described in Canalco's literature were used throughout this study.

Protein samples of 25 to 200μg were polymerized in a 3.5% gel at pH 6.8. An equal amount of a 3.5% stacking gel was subsequently poly-

merized on top of the sample gel. Finally, the separating gel at pH 9.0, containing 4, 6, 8 or 10% acrylamide by weight, was polymerized over this stacking gel. The sample and stacking gels were light-polymerized utilizing riboflavin as the free radical initiator while the separating gel was chemically polymerized with ammonium persulfate as the catalyst. A Tris-glycine buffer at pH 8.3 was used for the 1 to 1.5 hour electrophoretic runs which were carried out in a cold room at 4°. A constant current power supply was utilized to maintain a current of 3 milliamps per gel. A small amount of Bromphenol Blue was included in the upper cathode buffer compartment. This compound migrated as an ultra-sharp disc at the salt front and hence indicated the progress of the run. Excessive heat build-up or gel inconsistency resulted in warping or diffusing of this marker band.

The gels were stained for protein with Aniline Blue Black (0.5%) in 7% acetic acid or for IMP dehydrogenase activity with the tetrazolium reagent mixture previously described. Aniline Blue Black destaining was carried out electrophoretically at 12.5 milliamps per gel after a staining period of at least 1 hour. The stained gels were kept in 7% acetic acid.

The phosphocellulose purified preparations of highest specific activity showed four distinct protein bands migrating towards the anode (Figure 4). Two bands of similar intensity comprised about 80% of the total stained protein. The two minor bands moved slower than the two major bands. All four bands stained for IMP dehydrogenase activity. With heavier applications of protein (200 μg), four or five trace bands of protein were discernible moving faster than any of the dehydrogenase-containing bands. Reversing the electrodes in the Canalco apparatus showed there was no protein present which moved toward the cathode at this pH. When

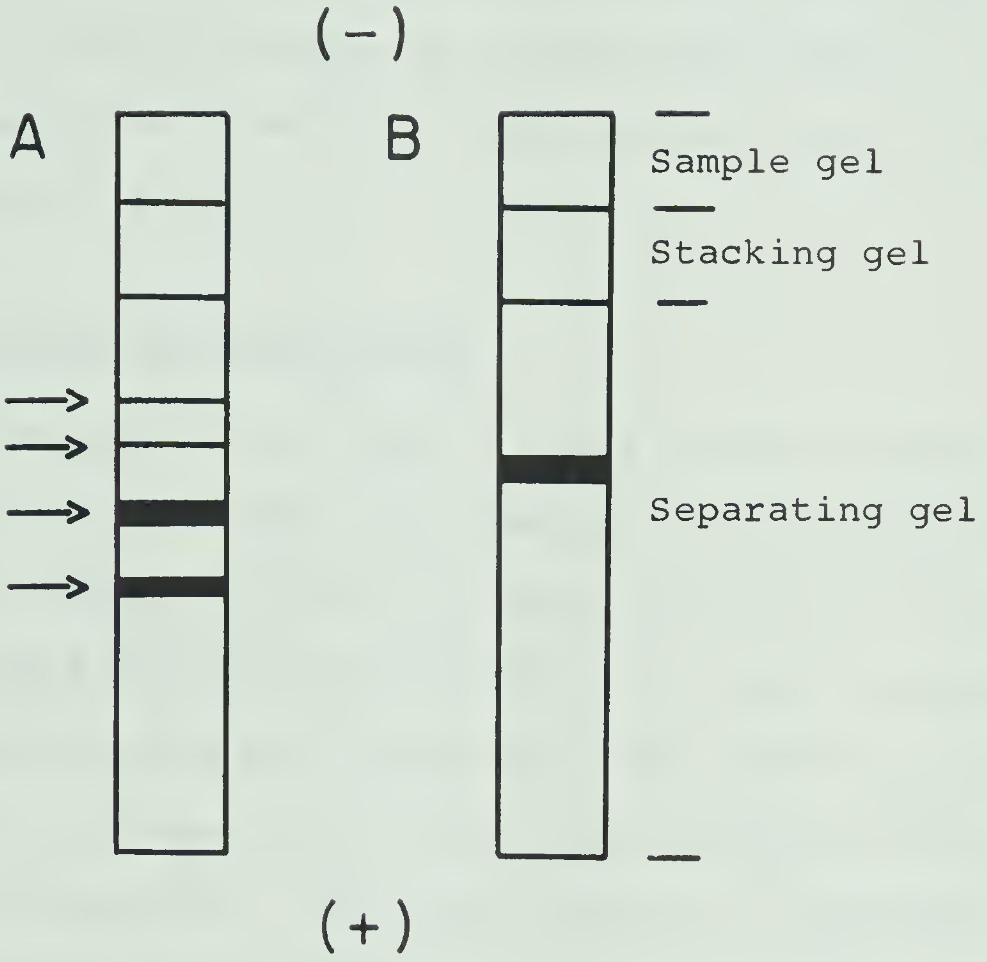

(−)
A
B
Sample gel
Stacking gel
Separating gel
(+)

Figure 4: Polyacrylamide electrophoresis of purified IMP dehydrogenase in a 10% gel. Gel A shows 4 protein bands (shown by arrows) which stain for IMP dehydrogenase activity. Gel B shows one protein band (no activity) when the same preparation is run in gels containing 3 M urea. The sample sizes were 60-70 μg of protein and runs were for 60' at 3 ma per gel.

[illegible]

the protein sample was applied in a 5% sucrose solution directly on the top of the separating gel, the same band pattern was obtained indicating that the polymerization process was not causing artifacts due to protein degradation.

As the porosity of the gel was increased (i.e., per cent acrylamide decreased), all four enzyme bands migrated farther in a given time, but none of the relative positions of the bands were altered. With any given gel, the mobilities of the protein bands was very nearly a linear function of the time of the run.

4. Sucrose Gradient Centrifugation

Sucrose gradients of 5-20% in 0.02 M potassium phosphate buffer of pH 7.4 were used initially. These gradients (4.5 ml) were kept at 4° for at least two hours prior to use, at which time 0.1 ml of a phosphocellulose-purified enzyme preparation was layered on the top of the gradient. A maximum of 200-250 μg of protein was applied. The centrifugation was carried out at 4° in the Beckman Model L centrifuge with the SW-39 rotor and speeds of 30 or 35 thousand rpm. After centrifugation, a 50% sucrose solution was forced into the bottom of the gradient tube by means of a hypodermic needle and syringe pump, and samples of 0.125 ml were collected from the top as the gradient was displaced upward through a specially designed flow-cell. These fractions were assayed by the standard assay for IMP dehydrogenase and also for protein by the Lowry method (48). Horse blood catalase ($s^{o}_{20,w}$ of 11.2 S) was used throughout as a reference protein.

The results of such an experiment are seen in Figure 5-A. The activity profile is skewed on the bottom side of the gradient and two main protein bands are apparent. Since the IMP dehydrogenase reaction is spe-

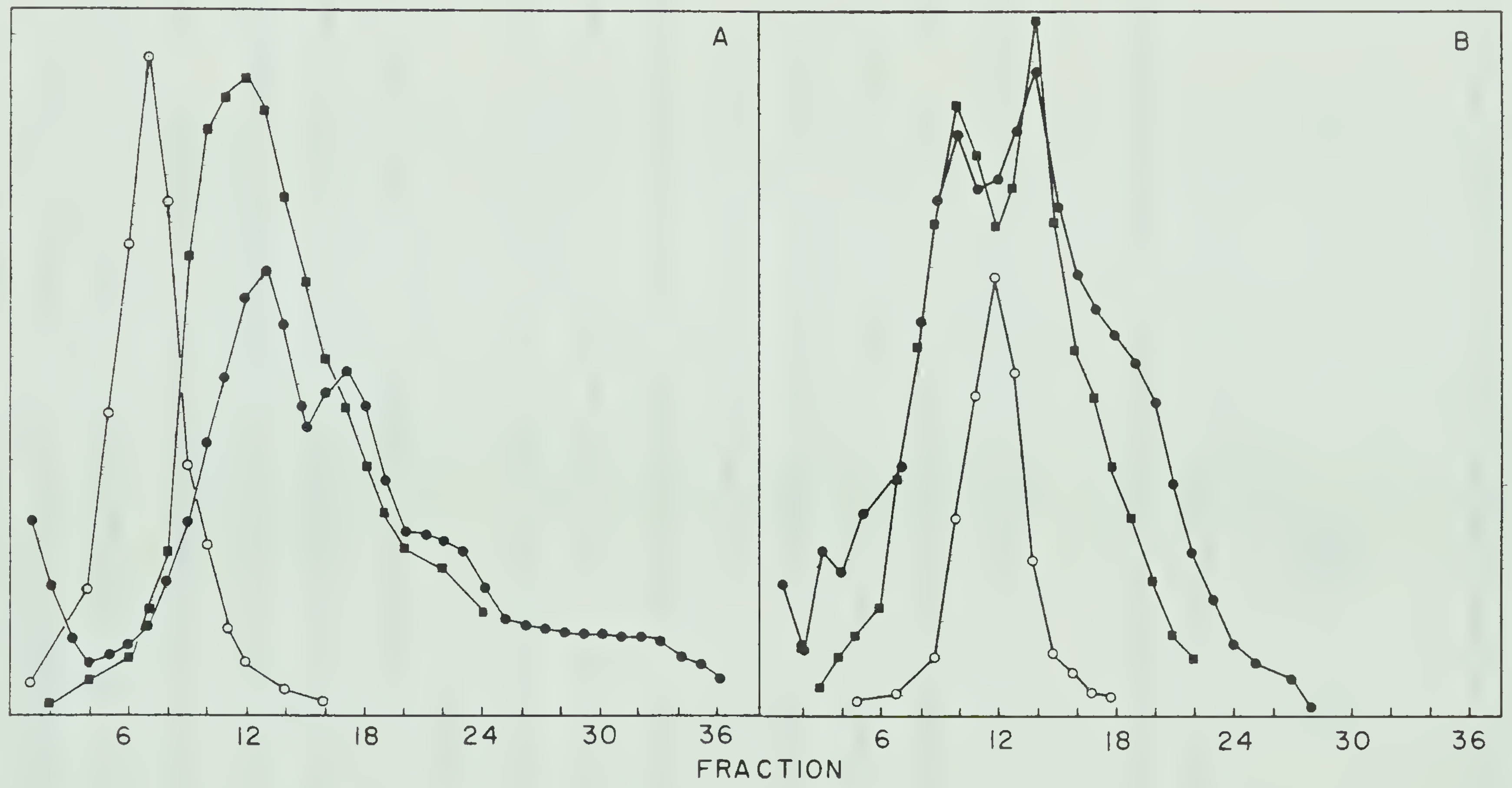


Figure 5: Sucrose gradient centrifugation of IMP dehydrogenase. Frame A; 5 hrs at 35K rpm in 5-20 % gradient at 4°. Frame B; 8 hrs at 35K rpm in 5-20 % gradient containing 0.1 M KCl at 4°. Protein is depicted by closed circles (●), IMP dehydrogenase activity by closed squares (■), and catalase activity by open circles (○).

cifically activated by potassium ion it was of interest to include 0.1 M KCl in the sucrose gradients. When this was done (Figure 5-B), two activity peaks which corresponded to two protein peaks were observed. The presence of KCl had no effect on the catalase mobility but greatly reduced the rate at which the IMP dehydrogenase peaks sedimented. The profiles were identical if catalase and the IMP dehydrogenase preparation were placed on the same or separate gradients. All of the protein applied to the gradients could be accounted for, together with 60-70% of the IMP dehydrogenase activity applied.

The migration of most proteins in 5-20% sucrose gradients is very nearly a linear function of time. It is thus possible to estimate the sedimentation coefficient for a protein by directly comparing the distances that the protein and a reference protein move from the meniscus under a given set of circumstances (49). With horse blood catalase (11.2 S) as the reference protein, values of 19.2 and 27.2 S were calculated for the protein peaks which were observed in the absence of KCl. With 0.1 M KCl present in the gradients the two peaks had sedimentation coefficients of 9.3 and 13.1 S.

A crude estimation of the molecular weight of a roughly spherical protein can be made from the sedimentation constant alone (50):

$$\frac{S_1}{S_2} = \left[\frac{MW_1}{MW_2}\right]^{2/3} \qquad (1)$$

where S_1 and MW_1 are for the uncharacterized protein and S_2 and MW_2 for the reference protein. Using values of 11.2 S and 250,000 for catalase (51), molecular weights of 560,000 and 945,000 (without KCl) or 190,000 and 315,000 (with KCl) may be calculated for the protein species present

in the IMP dehydrogenase preparations.

5. Analytical Ultracentrifugation

Some of the better phosphocellulose purified IMP dehydrogenase preparations have been examined with the Schlieren optical system in the Spinco Model E ultracentrifuge at 60,000 rpm. When feasible, protein fractions direct from the chromatographic columns were used, otherwise several such fractions were pooled and the protein precipitated with solid $(NH_4)_2SO_4$. The precipitate was redissolved in 0.025 M Tris-citrate (pH 8.1) containing 0.1 M KCl. The sedimentation runs were done at 8^o or 20^o in either a 4^o Kel-F cell or a 2.5^o double sector cell. An arbitrary value of 0.725 cm^3 per g has been assumed for the partial specific volume of IMP dehydrogenase.

These enzyme preparations consistently showed two major peaks of approximately equal areas. In some runs traces of two faster sedimenting components were also observed. The pattern was similar in the potassium phosphate buffer (pH 7.4) with 0.25 M KCl or in the Tris-citrate (pH 8.1) buffer with 0.10 M KCl. Likewise, 2×10^{-3} M GSH had no effect on the observed Schlieren pattern. Figure 6 shows the Schlieren pattern for the same enzyme preparation used in the sucrose gradient experiments of Figure 5. The $s_{20,w}$ values for the two major and one minor peaks were calculated (50) to be 9.1 S, 12.1 S and 15.1 S at a protein concentration of 0.2%. Centrifugation of other enzyme preparations at various protein concentrations gave $s_{20,w}$ values for the two major peaks of 6.3 and 9.1 S (1.0%); 7.1 and 9.9 S (0.4%); 8.3 and 11.4 S (0.3%).

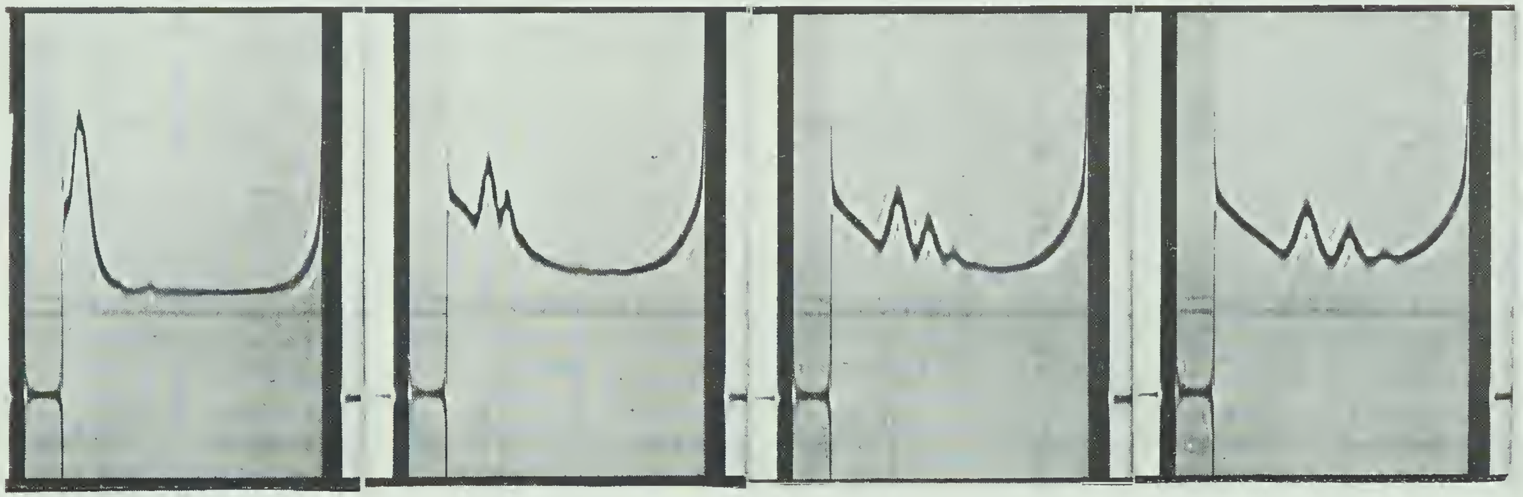


Figure 6: Ultracentrifugation of step 4 IMP dehydrogenase at 60,000 rpm and 8°. The protein (0.2%) was in a phosphate buffer (0.02M) at pH 7.4 containing 0.25 M KCl. The left frame was taken about 10 minutes after rotor reached full speed and the remaining frames were taken at 16 minute intervals. The bar angle was 50°.

6. Urea Treatment

The addition of urea to the standard assay mixture for IMP dehydrogenase resulted in the enzyme inhibition seen in Figure 7. The phosphocellulose purified enzyme was added last and the inhibition occurred within the 30 seconds required to start the assay. There was no indication of further inhibition during the 15-minute assay period.

If 0.1 ml of a complete 1 ml assay mixture containing urea (2.6M) was used as the source of enzyme in another assay mixture which contained no urea, the resulting inhibition was 16% that of the control (dilution of the enzyme 10X gives 10% of the initial rate). This dilution of enzyme also reduced the urea concentration to 0.26 M from 2.6 M. The inhibition by 0.26 M urea would be expected to be 17-18% (Figure 7), and it thus appears that this urea inhibition is completely reversed as quickly as it is established.

Ultracentrifugation of the IMP dehydrogenase under the conditions previously described (0.2% protein) but containing 3M urea resulted in the appearance of a single peak. After the corrections for temperature, viscosity and density were made, a $s_{20,w}$ of 6.4 S was obtained for this urea peak. This value may be slightly higher than the actual value for the protein alone because of urea binding to the protein. However, one can estimate, with the aid of equation 1, molecular weights of 105,000, 185,000, 290,000 and 390,000 for the urea peak and the three protein peaks of Figure 6.

The polyacrylamide gels used in the disc electrophoresis process were made 3 M in urea prior to the polymerization. When the IMP dehydrogenase was run in this system, a single band was evident after the gel was stained for protein. This new band did not correspond to any of the four bands which stained for IMP dehydrogenase activity as it ran slightly

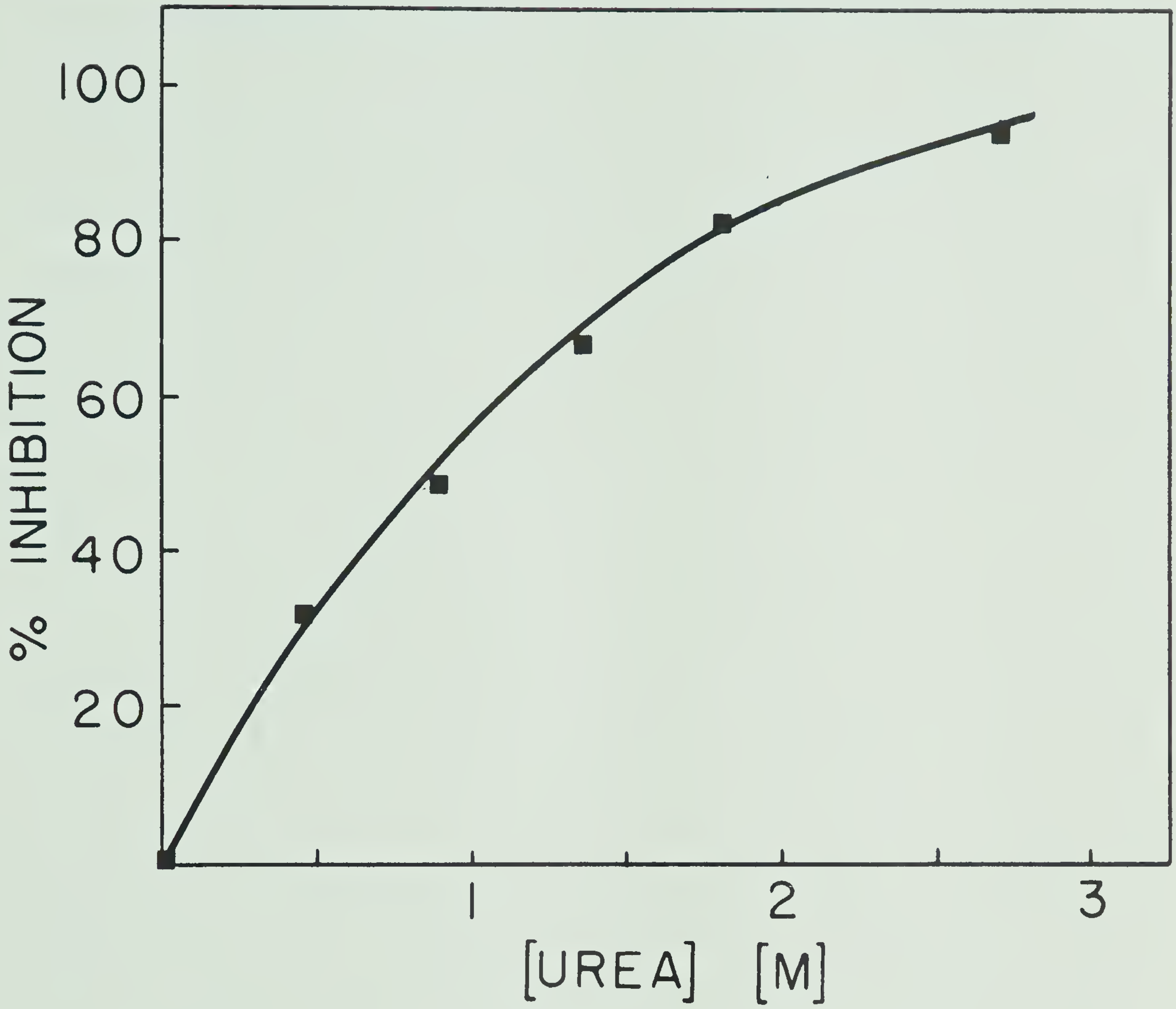


Figure 7: Urea inhibition of IMP dehydrogenase. The standard assay was used. IMP and enzyme were added last to the remaining assay components plus the various concentrations of urea shown.

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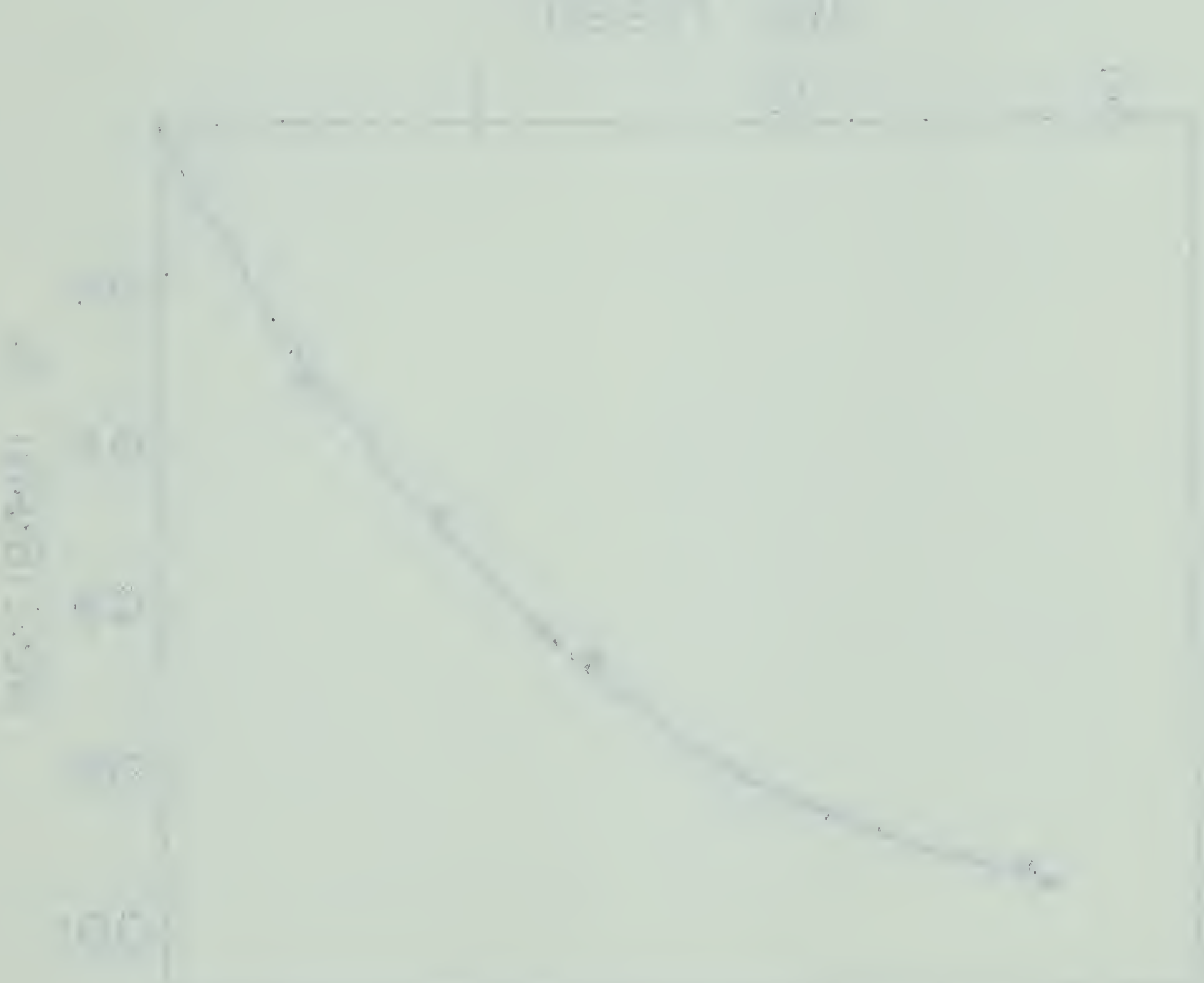

100

slower than the slowest of the two major bands. This urea band did not stain for dehydrogenase activity in the presence of the tetrazolium reagent mixture.

7. Discussion

Cellulose acetate electrophoresis but more specifically the polyacrylamide electrophoresis shows that the most highly purified enzyme preparations contain almost entirely protein that has IMP dehydrogenase activity. By visual examination of the stained polyacrylamide gels it is estimated that in excess of 80% of the total protein is found in two bands which are of approximately equal intensity. Migrating somewhat slower than these major bands are two additional bands which also have the dehydrogenase activity. The heavier sample applications gave rise to about 5 very weak protein bands which show the sample is not completely homogeneous. Again it is estimated visually that these additional bands would account for less than 5% of the total protein.

The question then arises as to whether isoenzymes or molecular aggregates account for these multiple bands. The relative intensity of the two major bands seemed in some way to be a function of the history of the sample, such as its age, number of times frozen, etc. This phenomenon was not systemically investigated. If there is equilibration between the molecular species seen in the disc electrophoresis, it is very slow compared to the length of a run (about 2 hours) since the bands were sharp with no evidence of streaking as one might expect if significant interconversion had occurred. Since the relative mobilities of these four enzyme bands remain essentially unchanged as gel porosity is decreased, it may be inferred that these bands are being separated more by charge differences than by the sieving effect of the polyacrylamide. This conclusion is however not entirely upheld by the sucrose gradient or ultracentrifugation experiments which indicate molecular species of different size are indeed present. The rather

good agreement in the sedimentation coefficients from sucrose gradients and ultracentrifugation experiments make it fairly certain one is dealing with the same molecular species in both cases. Furthermore, the skewing of the protein and activity profiles in the leading edge of the sucrose gradients may reflect the traces of faster sedimenting material which was often seen in the ultracentrifuge.

The effect of KCl on the sedimentation coefficients is probably an ionic strength effect, and does not reflect the entire stimulatory role played by potassium in this system. This conclusion is drawn from the observations that no change in sedimentation coefficients resulted when 0.1 M NaCl replaced the 0.1 M KCl in sucrose gradient experiments, or when the ultracentrifugation was done in the presence of 0.1 M Tris-citrate buffer instead of 0.1 M KCl. It was noted, though, that the specific activity of the 9.3 S sucrose peak was significantly lower when NaCl replaced KCl. This observation has not been further examined.

It is not completely certain whether the two peaks of the different centrifugation experiments correspond to the two major bands observed in the disc electrophoresis experiments. The Tris/HCl buffer concentration in the polyacrylamide separating gels is 0.38 M. One sucrose gradient containing a 0.38 Tris/HCl buffer indicated that the majority of IMP dehydrogenase activity sedimented with a coefficient of less than 9 S. It will be recalled that the presence of up to 0.25 M KCl in ultracentrifugation experiments had no apparent effect on sedimentation coefficients. More work must be done to see if this relatively high concentration of Tris is indeed breaking up the protein species present. The mobility of the two major protein bands seen in the polyacrylamide gels would indeed seem more likely if their molecular weights were less

than the 200,000 and 300,000 suggested by the centrifugation experiments. Canalco's technical literature shows that a molecular species of 300,000 molecular weight would have difficulty in entering their 10% gel, whereas the bands observed migrate readily in such a gel. One has then to consider that the polyacrylamide protein species may be some type of subunits of those seen in the centrifugation experiments.

Ultracentrifugation in 3 M urea gave rise to a single peak with a molecular weight, estimated from equation 1, of 105,000. This suggests that the molecular species of 9.1, 12.1 and 15.1 S seen in the ultracentrifuge may be dimers, trimers, and tetramers of this urea species. Disc electrophoresis showed only one band in the presence of urea and indicates a single electrophoretic species results upon the urea-induced dissociation. It is conceivable, though, that urea-induced conformational changes could mask out the charge differences which usually accompany different types of subunits. It is also possible that the protein species being separated on the polyacrylamide gels represent different electrophoretic protein moieties of 100,000 molecular weight. As the protein concentrations used in the assays are about one-hundredth that used in the centrifugation and electrophoresis experiments it is still impossible to decide which of these molecular species is active. The estimated protein concentration (47) in the major polyacrylamide bands is about 0.1 to 0.2% which is quite similar to that used in many of the centrifugation experiments.

In a Federation abstract, Powell *et al*. reported that the *E. coli* IMP dehydrogenase exhibited three activity peaks on Sephadex G-200 (22). They also observed a major peak and a faster sedimenting shoulder when the enzyme was examined in the ultracentrifuge at a protein concen-

tration of 0.35%. The major peak had a $s_{20,w}$ of 12.8 S. These results are similar to those described in this chapter for the enzyme from the closely related *A. aerogenes*.

IV. IMP DEHYDROGENASE KINETICS

1. Introduction

The conventional kinetic analysis of an enzyme system permits the selection of a kinetic model which is consistent with the observed initial velocity and product inhibition data. This kinetic model is a description of the order in which substrates react with the enzyme and the order in which products are released. If the reaction may be studied in both directions and if the absolute concentration of enzymic active centers may be determined, it may be possible to calculate all the individual rate constants and fully define the system in kinetic terms. More often though, only the individual dissociation constants may be determined.

The value of a kinetic model is perhaps two-fold. Firstly, it may give some indication as to whether substrate binding, catalysis or product release is the rate-limiting step. Selection of a kinetic model also allows actual dissociation constants to be calculated from the kinetic data. These dissociation constants describe directly substrate binding, whereas Michaelis constants usually do not.

Secondly, a kinetic model gives a frame of reference, uncertain though it may be, as to how the particular enzyme carried out its role in the intact cell. If for instance the kinetic parameters for an enzyme capable of catalyzing a reversible step are found to be such that they overwhelmingly favor the reaction in one direction, some insight has been gained as to the likely flow of metabolites along the pathway containing the enzyme in question.

A more specific value of a kinetic model is in drug design and

studies of drug mode of action. Drugs or antimetabolites may be designed for and tested against specific, purified enzymes in vitro. The effectiveness of the drug may be assessed directly as to the required result and concentration required for this result without complicating factors such as cell permeability and drug metabolism being involved.

Enzyme kinetics, like reaction kinetics in general, describe concentration effects and rates but often gives little insight into the electronic nature of the actual catalytic process. For example, one type of initial velocity kinetics, termed ping-pong kinetics by Cleland (40), suggest that some type of covalent intermediate is involved in catalysis. The absence of such kinetics does not, however, preclude the presence of such an intermediate. Knowledge of an intermediate of this type helps in postulating a chemical mechanism but still tells little as to how the catalytic efficiency of the enzyme is effected. Two problems in choosing a kinetic model for IMP dehydrogenase exist. The equilibrium is shifted so far to the side of XMP formation that it is not possible to study the reaction in both directions. As a result only half of the kinetic parameters may be evaluated. Certain parameters from a given kinetic model may be evaluated by studying either the forward or the reverse reaction. The agreement in such a case is often of use in the final selection of a satisfactory model.

There is at present still the uncertainty as to the source of the C-2 oxygen of XMP. It has been assumed (21) and is similarly assumed in this study that this oxygen arises directly from a water molecule. It is conceivable though, that it may be introduced in a two step process through, perhaps, an enzymic serine or tyrosine hydroxyl group. Such a group may actually provide the oxygen atom for XMP with water attacking

the enzyme amino acid in a concerted fashion to regenerate free enzyme. In a similar manner the $5'$-phosphate group may also be postulated to be the actual oxygen donor. This latter alternative is less likely due to the recent evidence that nucleotides exist in solution in the *anti* conformation (52) and might therefore be expected to bind in this form.

Although of interest mechanistically, the immediate oxygen source is not as important kinetically as is how water eventually participates in this reaction. The second problem is whether some form of a water molecule reacts directly from solution or first interacts with the enzyme in substrate-like fashion. One has then to consider either three substrate and two product kinetic models or two substrate and two product models. Making a final distinction between these alternatives is greatly complicated by the inability to study, in a straightforward manner, the reaction rate as a function of the water concentration.

It must always be kept in mind that the selection of a kinetic model is a process of elimination. All conceivable models must be examined as to whether or not their particular rate equations fit the experimental results. These rate equations can only disprove the selection of any given model and cannot definitively establish it, since it is always possible that some untried model will also be consistent with the data. This makes it very desirable to check the final selection of a model by independent methods such as physical binding studies or isotope exchange measurements.

The initial velocity data, at pH 8.1, for IMP dehydrogenase has already been reported from this laboratory (20). In an attempt to select a working kinetic model for the IMP dehydrogenase from *Aerobacter aerogenes*, product inhibition experiments were undertaken to complement

the already published initial velocity data at pH 8.1 (20). An important reason in obtaining a kinetic model was to determine if the actual dissociation constant for IMP may be calculated from kinetic experiments. This dissociation constant was of interest since a series of phosphate-analogues of IMP were being synthesized to study the role of the phosphate moiety in the binding of IMP to the dehydrogenase.

The pH optimum for IMP dehydrogenase is in the range 8.0 to 8.3 (20, 21), and drops quite sharply in more acid regions. Since the secondary ionization of the IMP phosphate group is about 6.3, it was of interest to see if lowering the pH had any effect on the various kinetic parameters. It was also possible that a change in pH could conceivably alter the kinetic model. To gain some insight into these questions a kinetic analysis was undertaken at pH 6.0. Lower pH values were not used since this would have necessitated using much higher protein concentrations and therefore complicated the comparisons by addition of an extra variable.

2. Methods

An IMP dehydrogenase preparation purified to the phosphocellulose stage was used throughout the subsequent experiments. The method of assay was similar to that described earlier with the addition of the sodium salts of NADH or XMP in the product inhibition experiments. Either a Tris-citrate buffer at pH 8.1 or a 2-(N-morpholino)ethane sulfonic acid buffer (65) at pH 6.0 was used, each at a final concentration of 0.050 M.

The reaction was started by addition of the IMP and the decrease in optical density was monitored at 340 mμ and 24° using a Cary Model 15 spectrophotometer with a full scale chart deflection of 0.10 OD.

The column chromatography fractions were diluted accordingly in

0.01 M Tris-citrate buffer (pH 8.1) containing glutathione (2×10^{-3} M) and KCl (0.1 M) to give the enzyme stock solution. These diluted solutions were made daily. From 0.5 to 1.0 μg of protein was normally used at pH 8.1, and 50 to 75 μg at pH 6.0. The pH 6.0 assays were linear with protein concentration from at least 25 to 125 μg of protein per assay.

When the data appeared to describe a linear relationship it was fitted to a straight line by the method of least squares assuming equal weighting to all points.

3. A Kinetic Model at pH 8.1

The initial velocity studies showed an intersecting pattern of lines when either the reciprocal of the IMP or NAD concentrations were plotted against the reciprocal of the velocity (20). These plots appeared to be linear, and hence the possibility of a completely random kinetic model in which the actual rates of enzyme-substrate interactions were rate-limiting was unlikely (40). The convergence of these lines, as opposed to parallel lines, suggested that the interaction of IMP and NAD with the enzyme proceeds in a sequential manner (40). In other words, none of the products can be released before all of the substrates are bound. The fact that this point of intersection was on or very close to the horizontal 1/S-axis is not in itself significant. It reflects a particular relationship between various kinetic constants but is dependent upon the kinetic model (40). This intersection on the 1/S-axis is often referred to by the statement that the binding of one substrate is independent of the binding of the other substrate.

To further the search for a suitable kinetic model, the inhibition of the forward reaction by XMP and NADH was examined. The effects of

these products on the reaction velocity when one of the substrates is varied at a fixed concentration of the other is seen in the accompanying figures. Figures 8 and 9 show that XMP is a competitive inhibitor of IMP but a noncompetitive inhibitor of NAD. Product inhibition by NADH is noncompetitive with respect to both IMP (Figure 10) and NAD (Figure 11). The replots of the slopes and intercepts from these figures which are used to calculate the various $K_{i\ slope}$ and $K_{i\ intercept}$ values (53) are seen to be linear except for the case of the intercepts from Figure 11.

Using the inspection techniques of Cleland (54), together with the above data, one may select three possible kinetic models (Figure 12) which could predict the observed initial velocity and product inhibition patterns. Model 1 is an ordered Bi Bi model (53) with IMP the first substrate to bind to the enzyme and XMP the last product to be released. Within this model, a water molecule or a hydroxyl ion would attack an activated IMP species directly from solution without necessitating any prior interaction with the enzyme.

Models 2-a and 2-b are extensions of Model 1 in which a water molecule acts as a substrate in that it binds to the enzyme, and it is either activated or specifically oriented in a manner facilitating the reaction. Models 1, 2-a, and 2-b could be interpreted mechanistically as addition of water across the 2,3 double bond of IMP associated with the transfer of the C-2 hydrogen to NAD. An elaboration of this overall mechanism has been previously presented (23), and postulates the activation of the purine C-2 for attack by hydroxyl ion by the addition of an enzymic sulfydryl group across the 1,6 double bond of IMP. The oxygen source, NAD, and IMP are considered to be present simultaneously on the enzyme so as to permit the catalytic process to proceed in a concerted fashion.

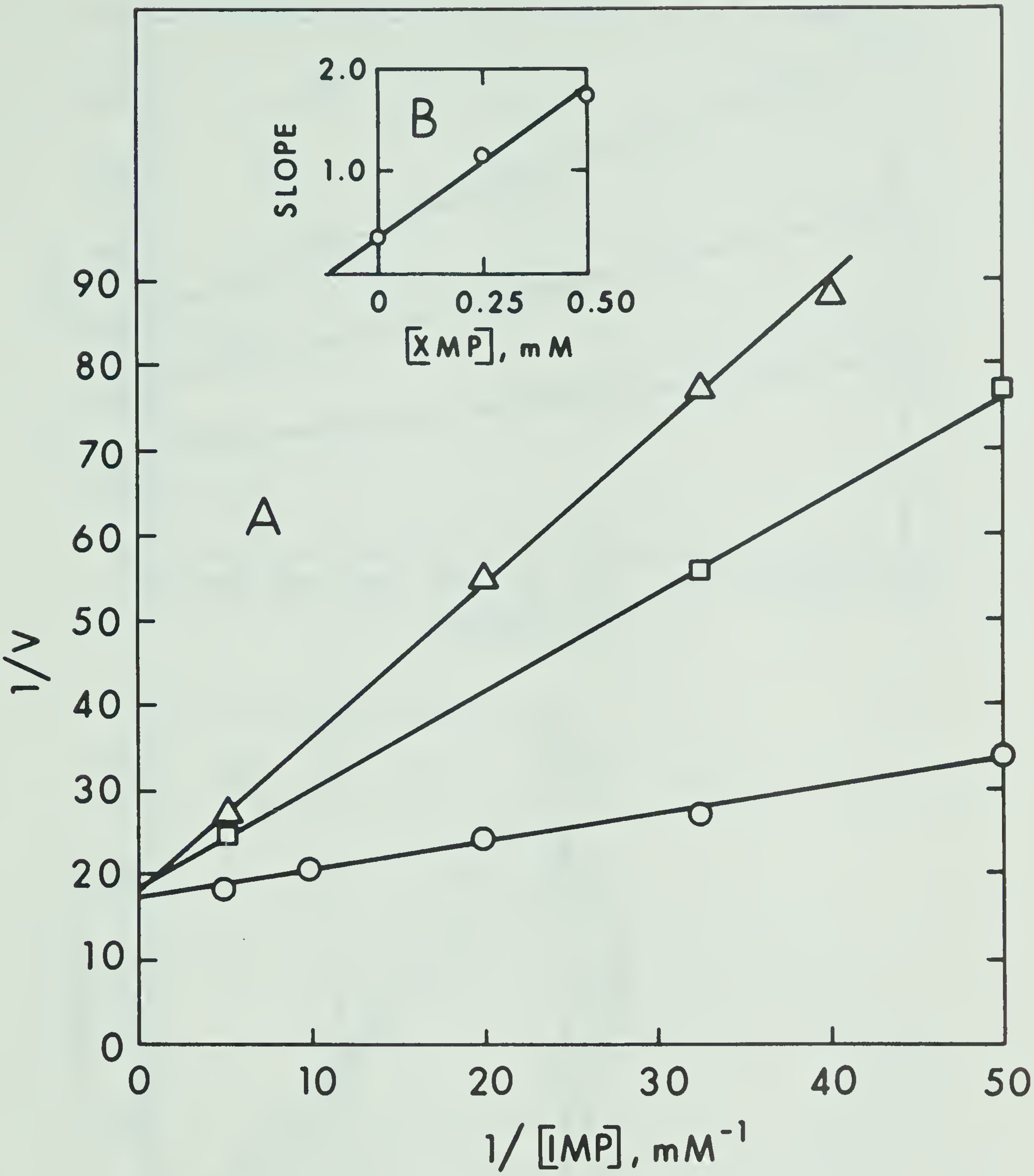


Figure 8: Frame A; reciprocal plot with IMP as variable substrate and XMP as product inhibitor of IMP dehydrogenase. NAD was 0.5 mM. Initial velocity is expressed as change in OD at 290 mμ per 10 minutes. XMP concentrations were: zero (○); 0.25 mM (□); 0.50 mM (△). Frame B; secondary plot of slopes from Frame A against XMP concentration.

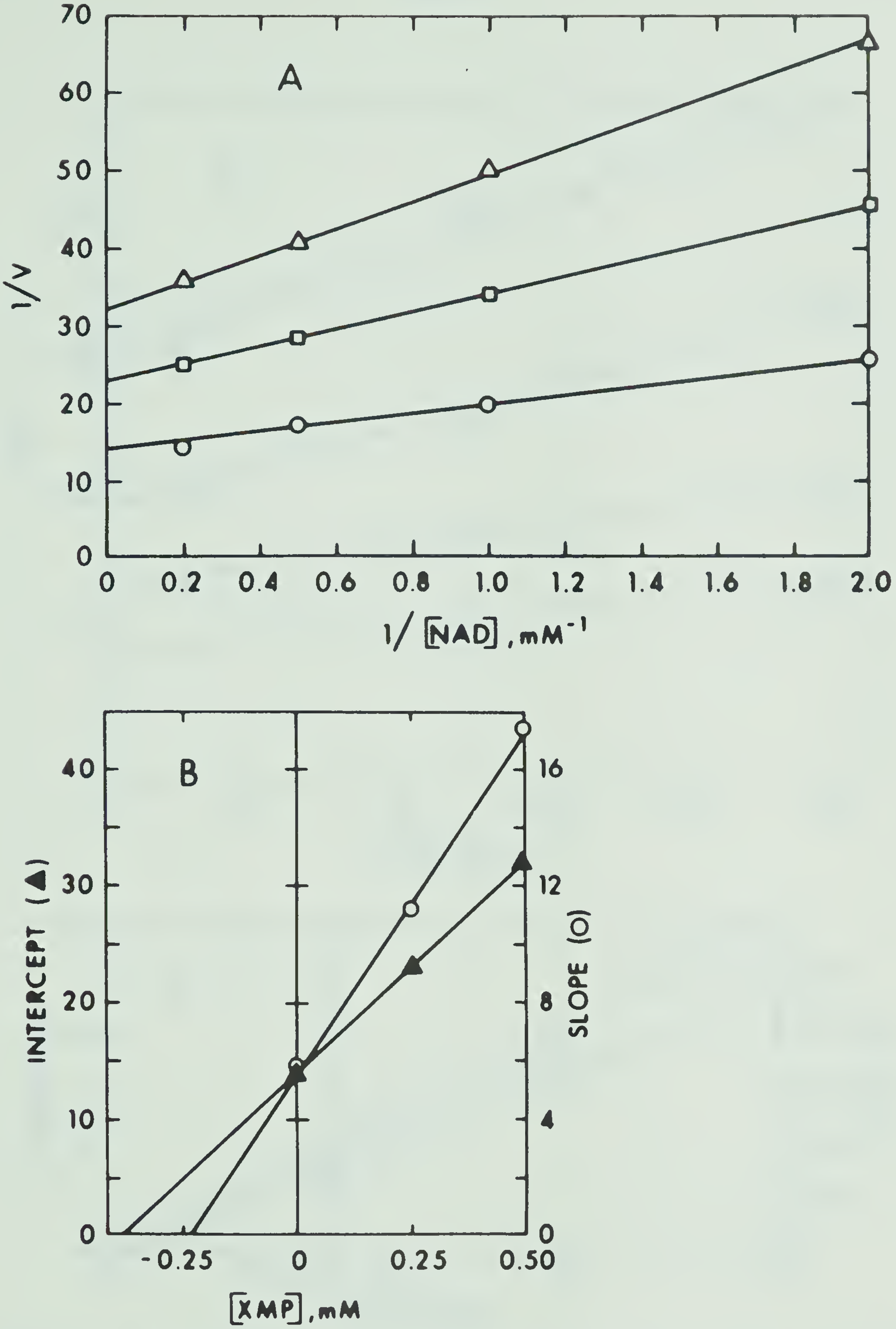


Figure 9: Frame A; reciprocal plot with NAD as variable substrate and XMP as product inhibitor of IMP dehydrogenase. IMP was 0.05 mM. XMP concentrations were: zero (○), 0.25 mM (□), and 0.50 mM (△). Frame B; secondary plots of slopes and intercepts from Frame A against the XMP concentrations.

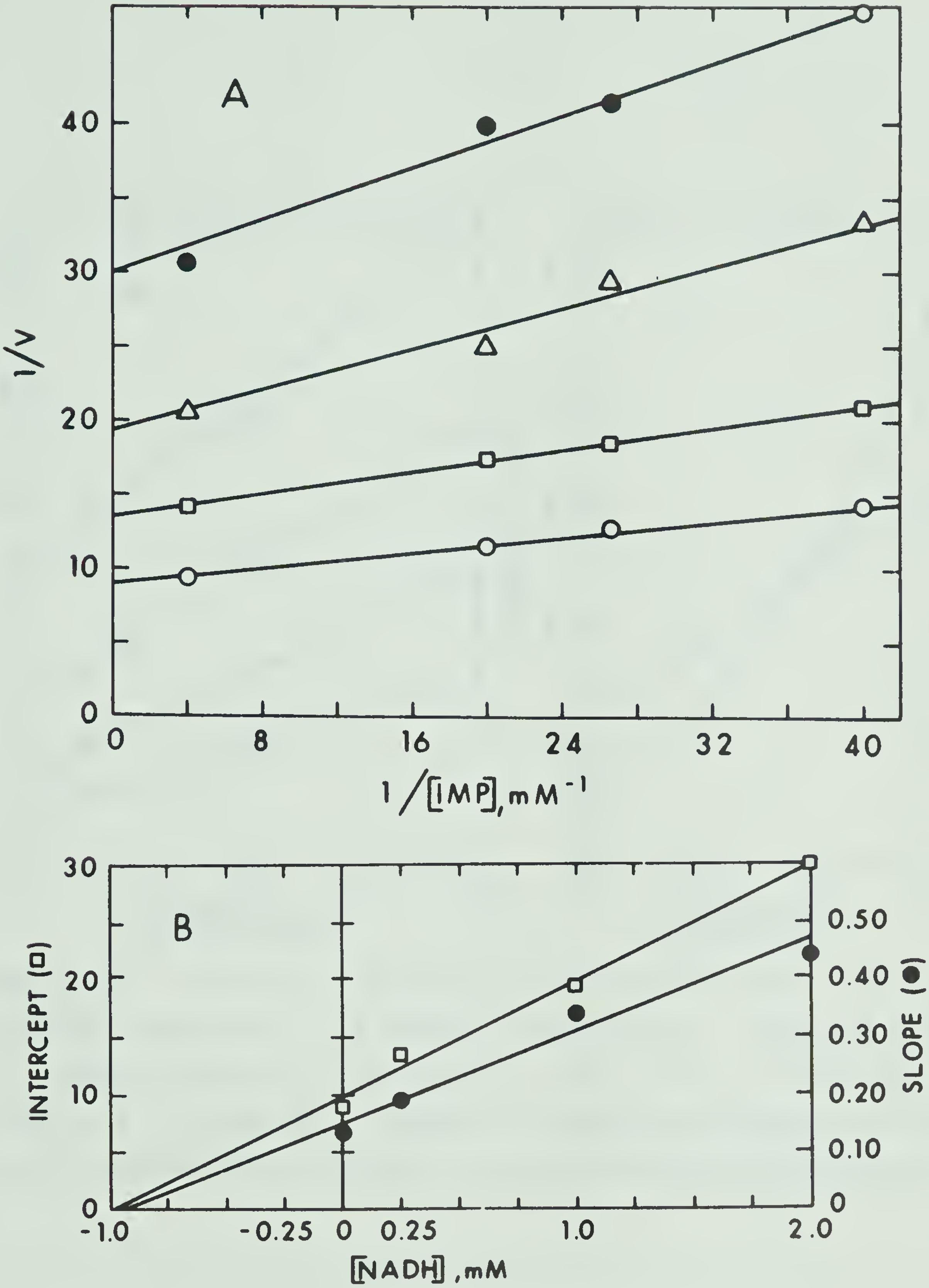


Figure 10: Frame A; reciprocal plot with IMP as variable substrate and NADH as product inhibitor of IMP dehydrogenase. NAD was 0.5 mM. NADH concentrations were: zero (○), 0.25 mM (□), 1.0 mM (△), and 2.0 mM (●). Frame B; secondary plots of slopes and intercepts from Frame A against NADH concentration

[illegible]

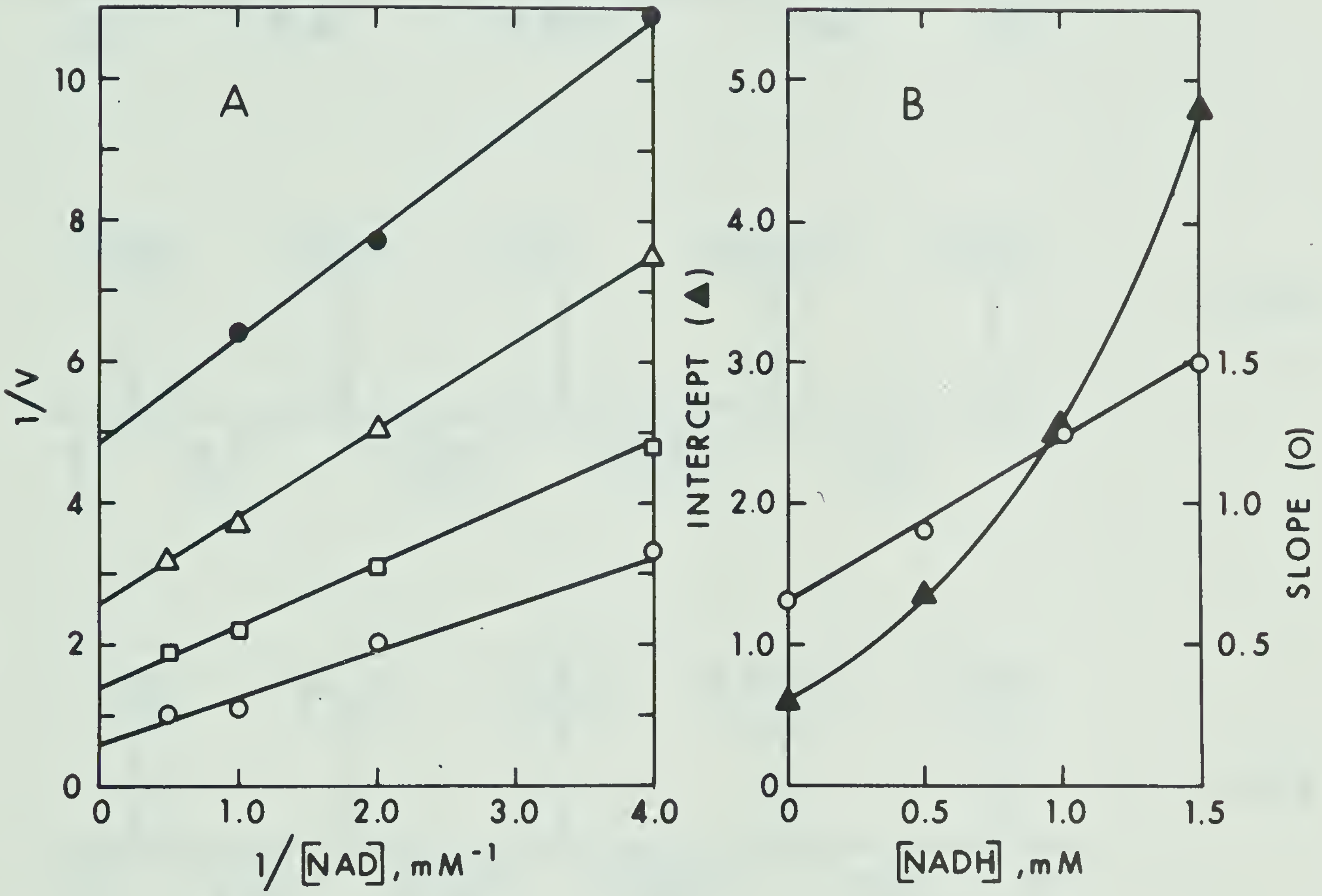


Figure 11: Frame A; reciprocal plot with NAD as the variable substrate and NADH as inhibitor. IMP was 0.25 mM. NADH concentrations were: zero (○); 0.5 mM (□); 1 mM (△); 1.5 mM (●). Frame B; secondary plots of slopes and intercepts from Frame A against NADH concentration.

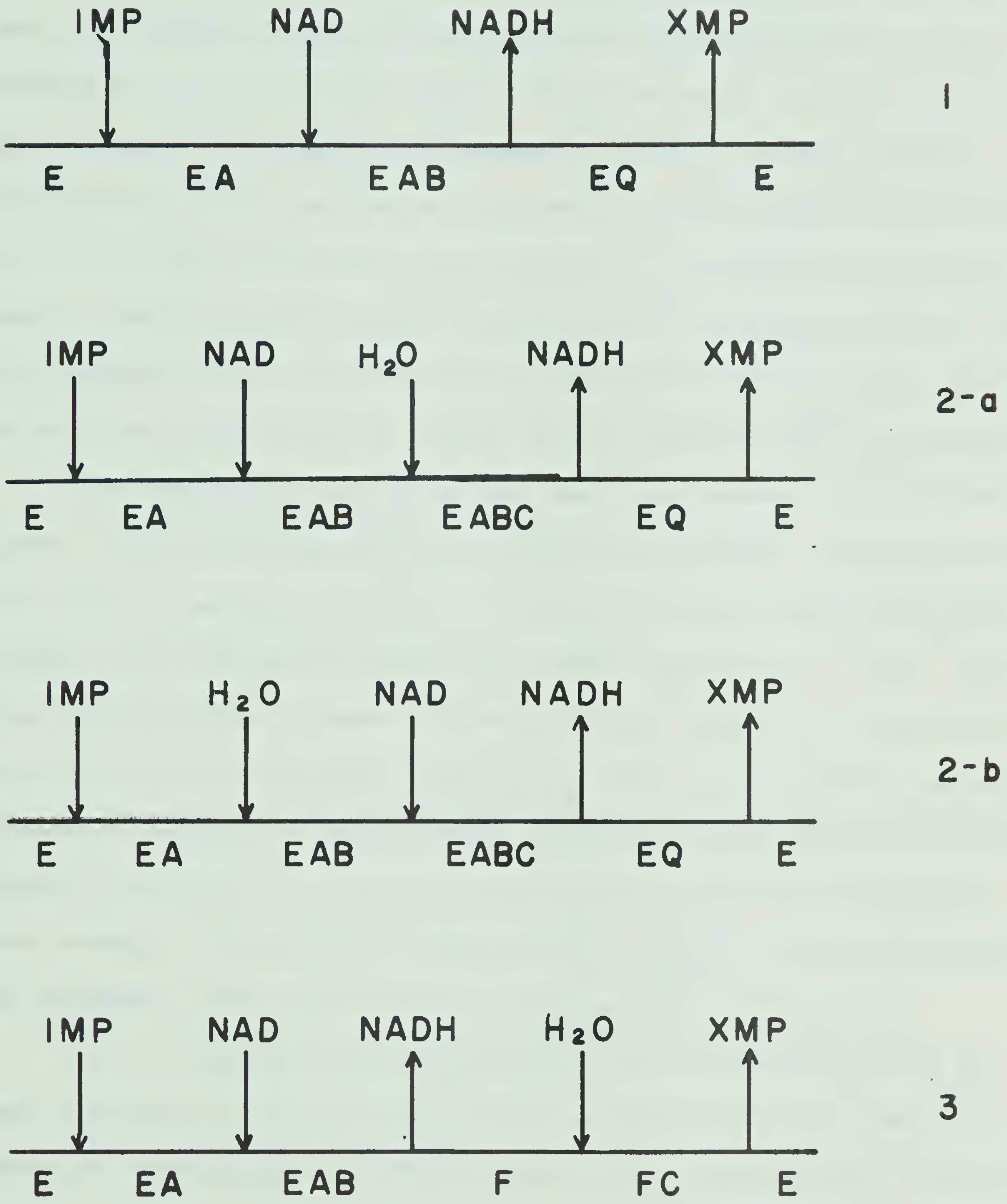

IMP
NAD
NADH
XMP
E
EA
EAB
EQ
E
1
IMP
NAD
H_2O
NADH
XMP
E
EA
EAB
EABC
EQ
E
2-a
IMP
H_2O
NAD
NADH
XMP
E
EA
EAB
EABC
EQ
E
2-b
IMP
NAD
NADH
H_2O
XMP
E
EA
EAB
F
FC
E
3

Figure 12: Possible kinetic models for IMP dehydrogenase. Models 1 and 2 are ordered Bi Bi and Ter Bi, respectively while Model 3 is a Bi Uni Uni Uni ping-pong model.

Model 3 is a Bi Uni Uni Uni ping-pong model (40) which is non-sequential since one of the products is released before the last substrate adds. This implies that an enzyme-IMP derivative may be formed during transfer of the C-2 hydrogen to NAD. Such a mechanism resembles that of glyceraldehyde 3-phosphate dehydrogenase in which an enzymic sulfydryl group forms an S-acyl derivative of glyceric acid 3-phosphate during transfer of the aldehyde hydrogen atom to NAD (55). By analogy, some reactive group on IMP dehydrogenase could form a bond at C-2 during transfer of the C-2 hydrogen to NAD, and the resulting enzyme-IMP derivative might then react with the oxygen source to produce XMP and regenerate the free enzyme.

The complete rate equations for these three models derived by the method of King and Altman (56) and expressed in Cleland's terminology (40) are shown in equations 2, 3 and 4 of Figure 13. The V_1 and V_2 represent maximal velocities in the forward and reverse direction; A, B, etc., are molar concentrations of substrates and products; K_a, K_p, etc., are the respective Michaelis constants; and K_{ia}, K_{ip}, etc., are additional constants required to express the rate equation in this relatively condensed form as opposed to leaving it in terms of the individual first and second-order rate constants. These kinetic parameters (K_a, K_{ia}, etc.) are functions of the individual first and second-order rate constants of Figure 12.

For the initial velocity studies, all terms containing P or Q become insignificant as one assumes product concentrations to be very low during the initial stages of the reaction. After dropping these P and Q terms, the remaining equation is placed in the reciprocal form, and then rearranged into the slope-intercept form of a straight line.

$$1/v = m/S + n \qquad (5)$$

In this equation v is the initial velocity at a substrate concentration

$$v = \frac{V_1V_2\left(AB - \frac{PQ}{K_{eq}}\right)}{K_{ia}K_bV_2 + K_bV_2A + K_aV_2B + V_2AB + \frac{K_qV_1P}{K_{eq}} + \frac{K_pV_1Q}{K_{eq}}} \qquad (2)$$

$$+ \frac{V_1PQ}{K_{eq}} + \frac{K_qV_1AP}{K_{ia}K_{eq}} + \frac{K_aV_2BQ}{K_{iq}} + \frac{V_2ABP}{K_{ip}} + \frac{V_1BPQ}{K_{ib}K_{eq}}$$

$$v = \frac{V_1V_2\left(ABC - \frac{PQ}{K_{eq}}\right)}{K_{ia}K_{ib}K_cV_2 + K_{ib}K_cV_2A + K_{ia}K_bV_2C + K_cV_2AB + K_bV_2AC} \qquad (3)$$

$$+ K_aV_2BC + V_2ABC + \frac{K_pV_1Q}{K_{eq}} + \frac{K_qV_1P}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{K_qV_1AP}{K_{ia}K_{eq}}$$

$$+ \frac{K_{ia}K_bV_2CQ}{K_{iq}} + \frac{K_qV_1ABP}{K_{ia}K_{ib}K_{eq}} + \frac{K_aV_2BCQ}{K_{iq}} + \frac{K_aK_{ic}V_2BPQ}{K_{ip}K_{iq}}$$

$$+ \frac{K_{ia}K_bV_2CPQ}{K_{ip}K_{iq}} + \frac{K_qV_1ABCP}{K_{ia}K_{ib}K_{ic}K_{eq}} + \frac{K_aV_2BCPQ}{K_{ip}K_{iq}}$$

$$v = \frac{V_1V_2\left(ABC - \frac{PQ}{K_{eq}}\right)}{K_{ia}K_bV_2C + K_cV_2AB + K_bV_2AC + K_aV_2BC} \qquad (4)$$

$$+ V_2ABC + \frac{K_pV_1Q}{K_{eq}} + \frac{K_qV_1P}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{K_qV_1AP}{K_{ia}K_{eq}}$$

$$+ \frac{K_{ia}K_bV_2CQ}{K_{iq}} + \frac{K_qV_1ABP}{K_{ia}K_{ib}K_{eq}} + \frac{K_aV_2BCQ}{K_{iq}}$$

$$+ \frac{K_aK_{ic}V_2BPQ}{K_{ip}K_{iq}} + \frac{K_aK_{ic}V_2BP}{K_{iq}}$$

Figure 13: Complete rate equations for the kinetic models of Figure 12 expressed in the terminology of Cleland. Equations 2, 3, and 4 correspond to kinetic models 1, 2, and 3, respectively.

of S. The m and n represent the slope and intercept of this 1/v vs. 1/S plot and are functions of the fixed substrate concentrations and various kinetic parameters. These slope and intercept terms for the kinetic models of Figure 12 are shown in Table II.

It can be seen from Table II that the method of replotting the intercepts from 1/v vs. 1/S plots in order to obtain the limiting Michaelis constants will not hold for Models 2 and 3. This results since the intercepts in these cases are functions of the third substrate concentration. It would thus be necessary to have initial velocity data at various water concentrations in order to determine the Michaelis constants for these models.

The complete rate equations of Figure 13 come into use for the product inhibition studies. As an example, the rate equation for the forward direction of the ordered Bi Bi model of Figure 12 in the presence of product P is obtained from equation 2 by dropping terms containing Q. When such equations are placed in reciprocal form and then rearranged into the slope-intercept form of equation 5, it is seen that three types of equations are obtained with additional expressions in either the slopes and/or the intercepts. In these equations v is the initial velocity with

$$\frac{1}{v} = m\left[1 + \frac{P}{K_{i\ \text{slope}}}\right]\frac{1}{S} + n\left[1 + \frac{P}{K_{i\ \text{intercept}}}\right] \qquad (6)$$

$$\frac{1}{v} = m\left[1 + \frac{P}{K_{i\ \text{slope}}}\right]\frac{1}{S} + n \qquad (7)$$

$$\frac{1}{v} = \frac{m}{S} + n\left[1 + \frac{P}{K_{i\ \text{intercept}}}\right] \qquad (8)$$

a given concentration of substrate (S) and product (P). The m and n terms are as described in Table II. The $K_{i\ \text{slope}}$ and $K_{i\ \text{intercept}}$ terms of

TABLE II: Slopes and Intercepts for the Possible Kinetic Models of IMP Dehydrogenase.

Model	Varied Substrate	Slope (m)	Intercept (n)
1	A	$\frac{K_{ia}K_b}{V_1B} + \frac{K_a}{V_1}$	$\frac{K_b}{V_1B} + \frac{1}{V_1}$
	B	$\frac{K_{ia}K_b}{V_1A} + \frac{K_b}{V_1}$	$\frac{K_a}{V_1A} + \frac{1}{V_1}$
2	A	$\frac{K_{ia}K_{ib}K_c}{V_1BC} + \frac{K_{ia}K_b}{V_1B} + \frac{K_a}{V_1}$	$\frac{K_b}{V_1B} + \frac{K_c}{V_1C} + \frac{K_{ib}K_c}{V_1BC} + \frac{1}{V_1}$
	B	$\frac{K_{ia}K_{ib}K_c}{V_1CA} + \frac{K_{ib}K_c}{V_1C} + \frac{K_{ia}K_b}{V_1A} + \frac{K_b}{V_1}$	$\frac{K_a}{V_1A} + \frac{K_c}{V_1C} + \frac{1}{V_1}$
	C	$\frac{K_{ia}K_{ib}K_c}{V_1AB} + \frac{K_{ib}K_c}{V_1B} + \frac{K_c}{V_1}$	$\frac{K_a}{V_1A} + \frac{K_b}{V_1B} + \frac{K_{ia}K_b}{V_1AB} + \frac{1}{V_1}$
3	A	$\frac{K_{ia}K_b}{V_1B} + \frac{K_a}{V_1}$	$\frac{K_b}{V_1B} + \frac{K_c}{V_1C} + \frac{1}{V_1}$
	B	$\frac{K_{ia}K_b}{V_1A} + \frac{K_b}{V_1}$	$\frac{K_a}{V_1A} + \frac{K_c}{V_1C} + \frac{1}{V_1}$
	C	$\frac{K_c}{V_1}$	$\frac{K_a}{V_1A} + \frac{K_b}{V_1B} + \frac{K_{ia}K_b}{V_1AB} + \frac{1}{V_1}$

these equations are again functions of fixed substrate concentrations and various kinetic parameters depending upon the kinetic model in question. These K_i expressions for the kinetic models of Figure 12 are tabulated in Table III. The types of inhibition described by equations 6, 7, and 8 are uncompetitive, competitive, and uncompetitive, respectively (53).

The data in Table III shows under what circumstances each of the proposed models of Figure 12 would predict the observed product inhibition patterns. The ordered Bi Bi Model 1 would predict the observed data without restriction. The reported initial velocity patterns showed reciprocal plots intersecting on or very close to the horizontal 1/S axis (20). As mentioned earlier this has no absolute significance but depends on the kinetic model describing the system in question. For Model 1, the conditions prerequisite to such an intersection may be determined by setting 1/v equal to zero in the reciprocal form of the initial velocity equation for this model. It can then be shown by simple algebra that K_a must equal K_{ia} if reciprocal plots are to intersect on the 1/S axis. Furthermore, the point of intersection when IMP is varied at different fixed concentrations of NAD will be equal to the negative reciprocal of the value for K_a and K_{ia}. It can also be shown that the point of intersection when NAD is varied at various fixed IMP concentrations will be the negative reciprocal of K_B. Replotting intercepts of these reciprocal plots should give the same values for the Michaelis constants as where obtained directly from the points of intersection. These values did in fact agree (20). In summary, it is seen that the Bi Bi Model 1 is consistent with the data and shows that the dissociation constant and Michaelis constant are both about 2×10^{-5} M.

Tables II and III show that Models 2 and 3 would not predict the

TABLE III: Kinetic Expressions for the K_i slopes and K_i intercepts for the Models of Figure 12.

Model	Varied Substrate	Product	K_i slope	K_i intercept
1	A (IMP)	Q (XMP)	K_{iq}	—
	B (NAD)	Q (XMP)	$\frac{K_{iq}(K_{ia} + A)}{K_{ia}}$	$\frac{K_{iq}(K_a + A)}{K_a}$
	A (IMP)	P (NADH)	$\frac{K_pK_{iq}}{K_q}\left[1 + \frac{K_aB}{K_{ia}K_b}\right]$	$\frac{K_pK_{ip}K_{iq}(K_b + B)}{K_bK_qK_{ip} + K_pK_{iq}B}$
	B (NAD)	P (NADH)	$\frac{K_pK_{iq}}{K_q}$	$\frac{K_{ip}(K_a + A)}{A}$
2	A (IMP)	Q (XMP)	K_{iq}	—
	B (NAD)	Q (XMP)	$K_{iq}\left[1 + \frac{K_{ib}K_cA + K_bAC}{K_{ia}K_{ib}K_c + K_{ia}K_bC}\right]$	$K_{iq}\left[1 + \frac{AC + K_cA}{K_aC}\right]$
	A (IMP)	P (NADH)	$\frac{K_{iq}K_p\left(K_{ia}K_{ib}K_c + K_{ia}K_bC + K_aBC\right)}{K_qK_{ia}K_{ib}K_c}$	$\frac{K_{iq}K_{ic}K_p\left(BC + K_{ib}K_c + K_cB + K_bC\right)}{K_qK_c\left(K_{ic}K_{ib} + K_{ic}B + BC\right)}$
	B (NAD)	P (NADH)	$\frac{K_pK_{iq}}{K_q}\left[1 + \frac{K_{ia}K_bC + K_bAC}{K_{ia}K_{ib}K_c + K_{ib}K_cA}\right]$	$\frac{K_{iq}K_pK_{ic}\left(AC + K_cA + K_aC\right)}{K_qA\left(K_cK_{ic} + K_cC\right)}$
3	A (IMP)	Q (XMP)	$\frac{K_{iq}}{1 + \frac{K_aK_{ic}B + K_{ia}K_bK_{ic}}{K_aBC + K_{ia}K_bC}}$	—
	B (NAD)	Q (XMP)	$\frac{K_{iq}\left(K_{ia}K_bC + K_bAC\right)}{K_{ia}K_bK_{ic} + K_{ia}K_bC}$	$\frac{K_{iq}\left(K_aC + K_cA + AC\right)}{K_aC + K_aK_{ic}}$
	A (IMP)	P (NADH)	$\frac{K_{ip}\left(K_{ia}K_bC + K_aBC\right)}{K_{ia}K_{ib}K_c}$	$\frac{K_{ip}\left(K_qBC + K_qK_bC + K_qK_cB\right)}{K_cK_qB + K_cK_{ib}K_a}$
	B (NAD)	P (NADH)	$\frac{K_{ip}\left(K_{ia}K_bC + K_bAC\right)}{K_{ia}K_{ib}K_c + K_{ib}K_cA}$	$\frac{K_{ip}C}{K_c}$

observed results in all cases. Only if the oxygen source involved in this reaction is unsaturating would Models 2 and 3 be consistent with the data.

If the oxygen source were saturating, Table II shows that Model 2-b would have predicted parallel lines in the initial velocity studies (20) as opposed to the observed intersecting pattern. This follows since the concentration of the oxygen source, substrate B in this model, being very large would dominate terms containing B and the other fixed substrate. It is seen that all slope terms which are functions of the fixed third substrate are also functions of the oxygen source concentration (B). Since a large B concentration masks these terms, no difference of slope is expected when IMP (A) is varied at different fixed concentrations of NAD (C) or vice versa. There will still be intercept effects as there are terms containing only IMP or NAD concentrations.

With Model 2-a it is seen from Table III that as the oxygen source concentration (C) becomes large the $K_{i\ slope}$ values obtained with NADH (P) as product inhibitor will also become large. Equation 6 shows that large $K_{i\ slope}$ values will decrease the effect of inhibitor on the slope term. Saturation by C would then predict NADH to be an uncompetitive inhibitor of IMP or NAD and not a noncompetitive one (Figure 10, 11).

Similarly with Model 3, a large oxygen source concentration (C) would result in large $K_{i\ slope}$ and $K_{i\ intercept}$ values when NADH was used as an inhibitor (Table III). This then predicts that NADH would not inhibit the reaction, whereas Figures 10 and 11 show that it does. The same conclusion can be easily predicted from the schematic of Model 3 (Figure 12). As NADH and the oxygen source both combine with the same enzyme form, a saturating concentration of C would leave none of the enzyme for NADH to bind to, and hence the presence of NADH would have no

effect on the reaction velocity.

The Models 2-a, 2-b and 3 are then consistent with the kinetic data only if the oxygen source is unsaturating. This source could be a water molecule itself or perhaps a hydroxyl ion. It would seem unlikely to this writer that a hydroxyl ion would specifically bind to an enzyme prior to catalysis, or in fact that such a binding would have any advantage since this species is already very reactive. It is perhaps easier to understand why a water molecule may be specifically bound since electron donors on the enzyme may hydrogen bond to the water hydrogens, and in this manner provide a more reactive oxygen for nucleophilic attack. If such was the case, one might think that the aqueous assay medium, having a water concentration in excess of 50 M, would be sufficient to saturate the enzyme. From this type of argument the ordered Bi Bi model seems a more likely choice for the IMP dehydrogenase system.

The observation that the initial velocity reciprocal plots intersected on the horizontal axis (20) is not consistent with Model 2-b as it is difficult to find conditions that would give such a pattern. Models 2-a and 3 would give reciprocal plots intersecting on the 1/S axis if K_{ia} equaled K_a and the oxygen source was saturating. It has been previously shown that if the oxygen source was saturating in Models 2-a and 3 the predicted results would not be consistent with those obtained experimentally. Model 1 becomes again the best choice for IMP dehydrogenase.

Using the ordered Bi Bi model, various additional kinetic parameters emerge from the kinetic data. The dissociation constant of IMP is then about 2.0×10^{-5} M, the same as the IMP Michaelis constant, while the dissociation constant for XMP is 1.2×10^{-4} M. The reported K_i for GMP with the IMP dehydrogenase from A. aerogenes is 1.3×10^{-4} M (20) and this

then represents the dissociation constant for GMP. GMP has subsequently been found to be a competitive inhibitor of IMP with this enzyme from Sarcoma 180 tumor cells (19) and from E. coli (22). The reported K_i values were 3×10^{-4} M and 1.8×10^{-4} M, respectively. The observation that IMP was 5 to 6 times more effective than GMP in protecting the IMP dehydrogenase of A. aerogenes from the inactivation incurred when 6-chloropurine nucleoside 5'-phosphate was preincubated with the enzyme (20) is then consistent with the dissociation constants for IMP and GMP. The internal consistency of a particular model may be checked by comparing the numerical values of kinetic parameters which may be determined by either initial velocity or product inhibition experiments. For instance, using the product inhibition value for K_{iq} of 1.2×10^{-4} M and the expression for the $K_{i\ intercept}$ when NAD is varied with XMP as inhibitor (Table III), it is possible to evaluate K_a. The numerical value for this K_i is 3.8×10^{-4} M and is obtained from Figure 9. With this data a value of 2.3×10^{-5} M may be calculated for K_a which is in good agreement with the value of 2.0×10^{-5} M obtained from the initial velocity data. Calculation of kinetic parameters in this manner is subject to greater error since the values of the other kinetic constants used in such a computation are each subject to error and hence give rise to a large accumulative error.

Dehydrogenases have been perhaps studied kinetically more than any other class of enzymes. From these numerous studies certain features emerged. While both random and ordered kinetic models have been found to describe various systems, it appears that the first substrate to bind in ordered models is the pyridine coenzyme. Horse liver alcohol dehydrogenase is believed to utilize predominately ordered substrate binding

with initial coenzyme binding (58), although, isotope exchange studies show that a small percentage of the molecular conversion proceeds via initial aldehyde or alcohol binding (59). Kinetic studies were not sensitive enough to detect this small amount of random character. Lactic dehydrogenase (60), malic dehydrogenase (decarboxylating) (61) and Neurospora NAD-isocitrate dehydrogenase (62) are other examples of dehydrogenases believed to employ ordered models with initial coenzyme binding. While the lactic dehydrogenase appears to have ordered binding at pH 7.9, it does not have completely ordered binding at pH 9.7 (60). The muscle glyceraldehyde-3-phosphate dehydrogenase is an example of a dehydrogenase which permits random substrate binding (63).

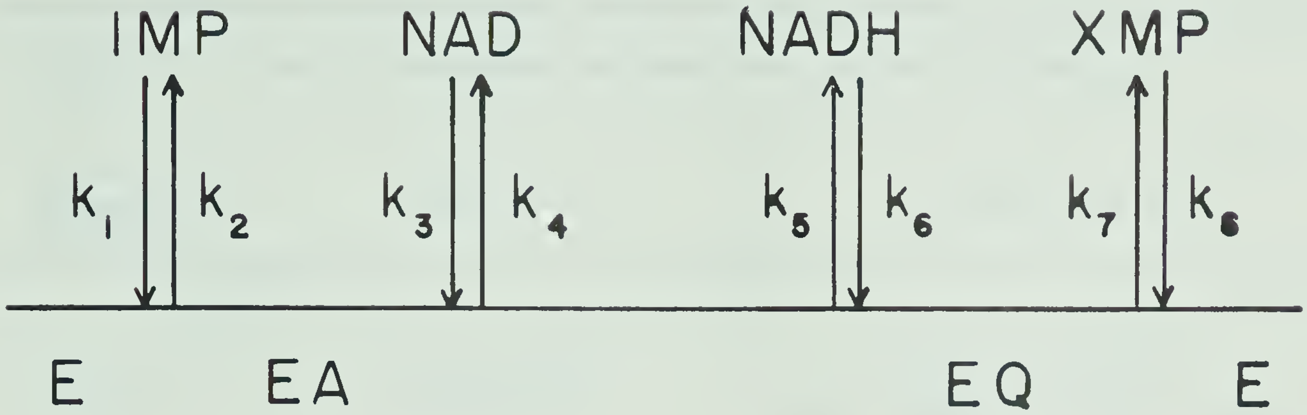
Recent studies on the forementioned enzymes as well as many others using the more sensitive isotope exchange experiments (64) tends to indicate that absolute ordered binding is only the limiting case of the more general random binding situation. Under certain conditions of temperature, pH, etc. one pathway of the random case is more favorable from an energy viewpoint. The determination of whether absolute ordered binding is involved in any given system will ultimately depend on the sensitivity of the measurements involved.

IMP dehydrogenase appears to follow a predominately ordered binding sequence at pH 8.1. Unlike the vast majority of other dehydrogenases, the pyridine coenzyme is not the first substrate bound in this system. The significance of such an observation, if any, is unknown. A preliminary report on the kinetic studies of IMP dehydrogenase from Sarcoma 180 suggests that this enzyme too requires ordered binding with IMP the first substrate to add to the enzyme (24).

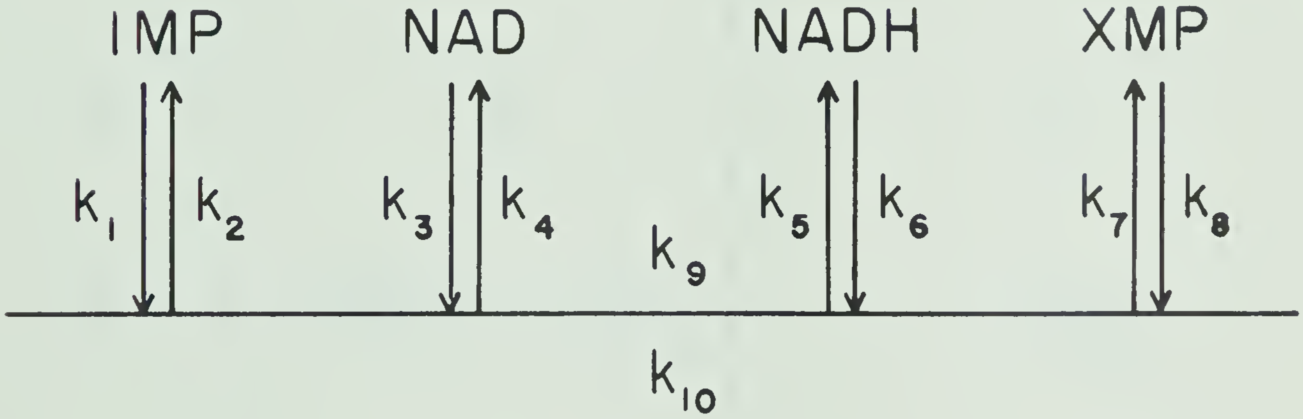
4. Expansion of the Kinetic Model

The kinetic models shown in Figure 12 are the stylized versions of each case. They contain only the rate constants which describe the addition of substrates or the release of products. There is no consideration built into these models for the actual catalytic oxidative process effected by IMP dehydrogenase. One might expect that in the more rigorous case there should be additional rate constants involved which would describe the rate of the actual molecular interconversion of IMP and NAD to XMP and NADH. The simple ordered Bi Bi model and an expanded model are shown in Figure 14.

It is seen that only two additional rate constants have been introduced to describe the chemical interconversion. It might be argued that since water is postulated to react directly with the ternary complex, at least two sets of constants should be included, one describing the rate of the attack by water and a second describing the oxidation of the resulting intermediate. This may be true, but additional enzyme isomerizations each requiring a set of constants may also occur, and it is impossible to know how far to pursue this line of reasoning. The two additional constants of Model 1-a (Figure 14) describe everything that occurs from the formation of the EAB complex till the EPQ complex starts to dissociate to EQ and P. The derivation of the rate equations for this more complex model gives rise to equations which are identical in form to that shown in Table II for the simple ordered Bi Bi mechanism. What has changed is that some of the kinetic parameters such as K_a, K_b, etc. are now more complex functions of the individual rate constants involved. Table IV shows for Model 1 and 1-a certain pertinent kinetic parameters expressed in terms of the individual rate constants for the two models in question. The K_{ia} and K_{iq}


IMP
NAD
NADH
XMP
k_1
k_2
k_3
k_4
k_5
k_6
k_7
k_8
E
EA
EQ
E

MODEL I


IMP
NAD
NADH
XMP
k_1
k_2
k_3
k_4
k_9
k_5
k_6
k_7
k_8
k_{10}

MODEL I-a

Figure 14: The ordered Bi Bi and expanded Bi Bi kinetic models showing the individual first and second-order rate constants for the addition and release of substrates and products.

TABLE IV: Kinetic Parameters as Functions of Rate Constants.[a]

Kinetic Parameter	Simple Bi Bi	Expanded Bi Bi
$V_{forward}$[b]	$\frac{k_5\ k_7\ [E_t]}{(k_5 + k_7)}$	$\frac{k_9\ k_5\ k_7\ [E_t]}{(k_5k_7 + k_7k_{10} + k_9k_7 + k_9k_5)}$
$V_{reverse}$	$\frac{k_2\ k_4\ [E_t]}{k_2 + k_4}$,	$\frac{k_{10}\ k_2\ k_4\ [E_t]}{(k_4k_{10} + k_2k_{10} + k_9k_2 + k_2k_4)}$
K_{ia}	$\frac{k_2}{k_1}$	$\frac{k_2}{k_1}$
K_{iq}	$\frac{k_7}{k_8}$	$\frac{k_7}{k_8}$
K_a	$\frac{k_5\ k_7}{k_1\ (k_5 + k_7)} = \frac{V_f}{k_1\ [E_t]}$	$\frac{V_f}{k_1\ [E_t]}$
K_b	$\frac{k_7\ (k_4 + k_5)}{k_3\ (k_5 + k_7)}$	$\frac{k_7\ (k_9k_5 + k_5k_4 + k_4k_{10})}{k_3\ (k_5k_7 + k_7k_{10} + k_9k_7 + k_9k_5)}$

[a] Individual rate constants as in Figure 7.

[b] Maximum velocity in forward direction.

are the only parameters which remain unaltered.

Various things become apparent when the rate constant equivalents for the various kinetic parameters are examined for this Bi Bi model. The maximum velocity in the forward direction is a function of the concentration of active enzymatic sites, the rate of molecular interconversion, and also the rate of dissociation of NADH and XMP. The Michaelis constant for IMP is dependent upon the maximal velocity and the rate at which IMP and enzyme interact. It is easily seen in this manner that using the Michaelis constant for IMP as an indication of binding capacity is not satisfactory. Similarly, studying the effect of pH on K_a and attempting to correlate changes with actual binding is of limited value since corrections for pH effects on maximal velocity and rates of dissociation on NADH and XMP must first be introduced. The Michaelis constants are by definition the molar concentrations required to give half maximal velocity when all other substrates are saturating. They should then be independent of terms concerned with addition of the other substrates to the enzyme. This is indeed seen in Table IV as there is no k_3 constant in the expression for K_a and no k_1 in that for K_b.

The reaction catalyzed by IMP dehydrogenase is essentially irreversible. From Table IV it is seen this could be caused by either k_{10}, k_2 or k_4 being very low or by a very poor affinity of the enzyme for XMP and/or NADH. The product inhibition experiments rule out the latter possibility. If k_2 or k_4 were limiting (i.e., very small) it should not be possible to inhibit this reaction by adding to an assay mixture containing saturating amounts of IMP any concentration of a competitive inhibitor of IMP (similarly for NAD and competitive inhibitors), since there would be virtually no dissociation of the substrate and no chance for inhibitor

binding. Since this too is not the case, one is left with the alternative that molecular interconversion or some other process between the formation of EPQ and the subsequent formation of EAB is rate limiting in the reverse direction.

5. Kinetics at pH 6.0

Figure 15 and 16 show the initial velocity data, plotted in reciprocal form, for IMP dehydrogenase at pH 6.0. It is seen that as at pH 8.1 an intersecting pattern of lines is obtained and that slopes and intercepts are linear functions of the reciprocal of the fixed substrate concentrations. It is apparent, however, that the point of intersection for these reciprocal plots is in the upper, left-hand quadrant and not on the horizontal 1/S axis as was the case at pH 8.1 (20).

One might think of an ordered Bi Bi model as a limiting case of the more general random addition situation. That is, due to thermodynamic reasons one pathway in the random case is much more favored than the other. A change in environment such as temperature or pH may then overcome this energy barrier so that the order of substrate-enzyme interaction may change. As a consequence, before any pH effects are assessed, it must be reasonably well established that the comparisons being made are valid ones. Complete product inhibition data was not obtained at pH 6.0 but Figure 17 shows that XMP is a linear noncompetitive inhibitor of NAD. This was also the case at pH 8.1. If the kinetic model had become random one would have expected competitive inhibition (40). Hence, it is concluded that the ordered Bi Bi model may still be used at this lower pH.

The numerical values for various of the kinetic parameters of IMP dehydrogenase at pH 6.0 together with their equivalent values at pH 8.1

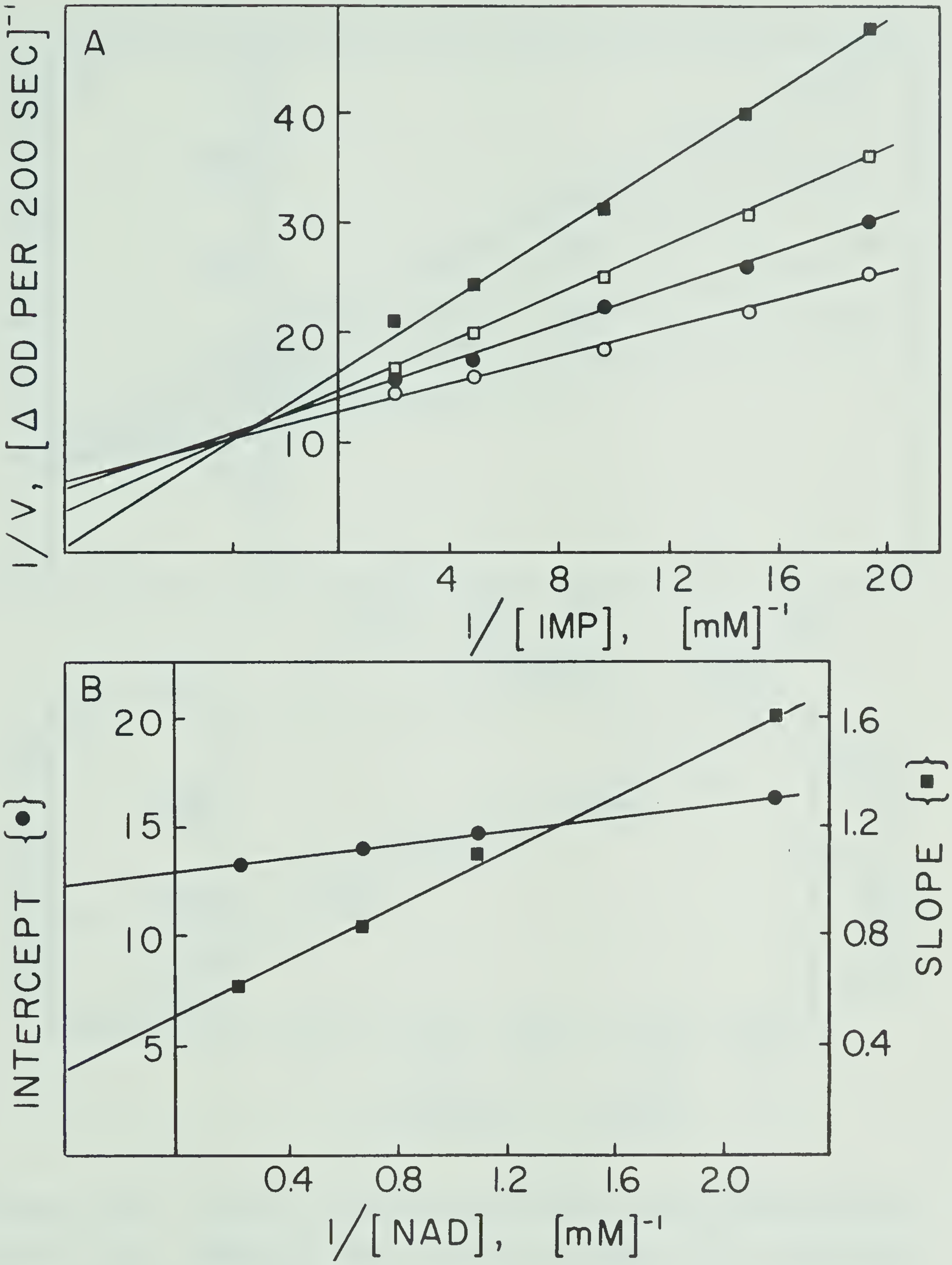


Figure 15: Initial velocity data for IMP dehydrogenase at pH 6.0. Frame A; IMP varied at fixed NAD concentrations of 4.6×10^{-4} M (■), 9.2×10^{-4} M (□), 1.5×10^{-3} M (●), and 4.6×10^{-3} M (○). Frame B; replots of the slopes and intercepts vs. the reciprocal of the NAD concentration. OD changes measured at 340 mμ.

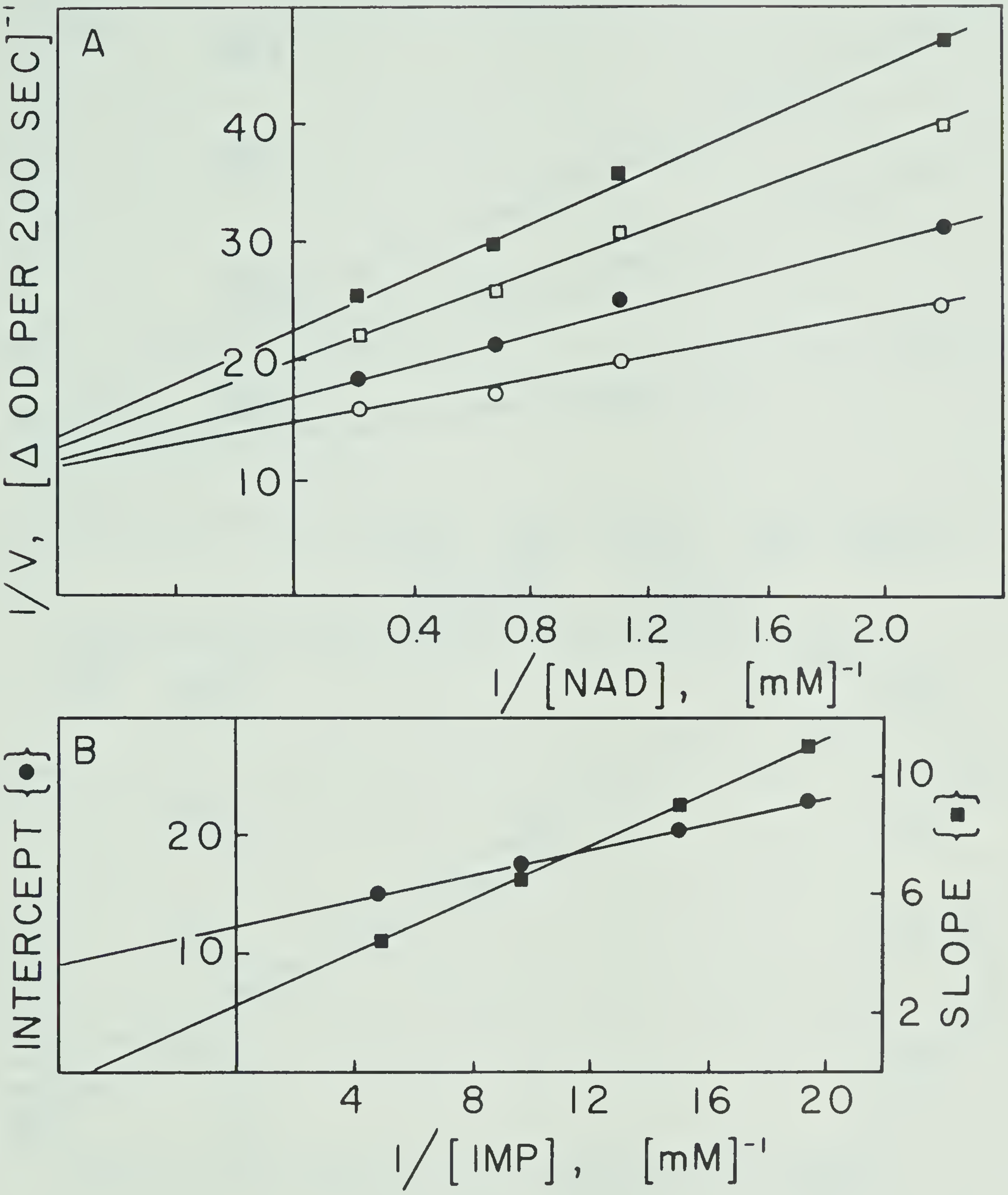


Figure 16: Initial velocity data for IMP dehydrogenase at pH 6.0. Frame A; NAD varied at fixed IMP concentrations of 5.2 x 10^{-5} M (■), 6.7 x 10^{-5} M (□), 1.0 x 10^{-4} M (●), and 2.1 x 10^{-4} M (○). Frame B; replots of the slopes and intercepts vs. the reciprocal of the IMP concentration.

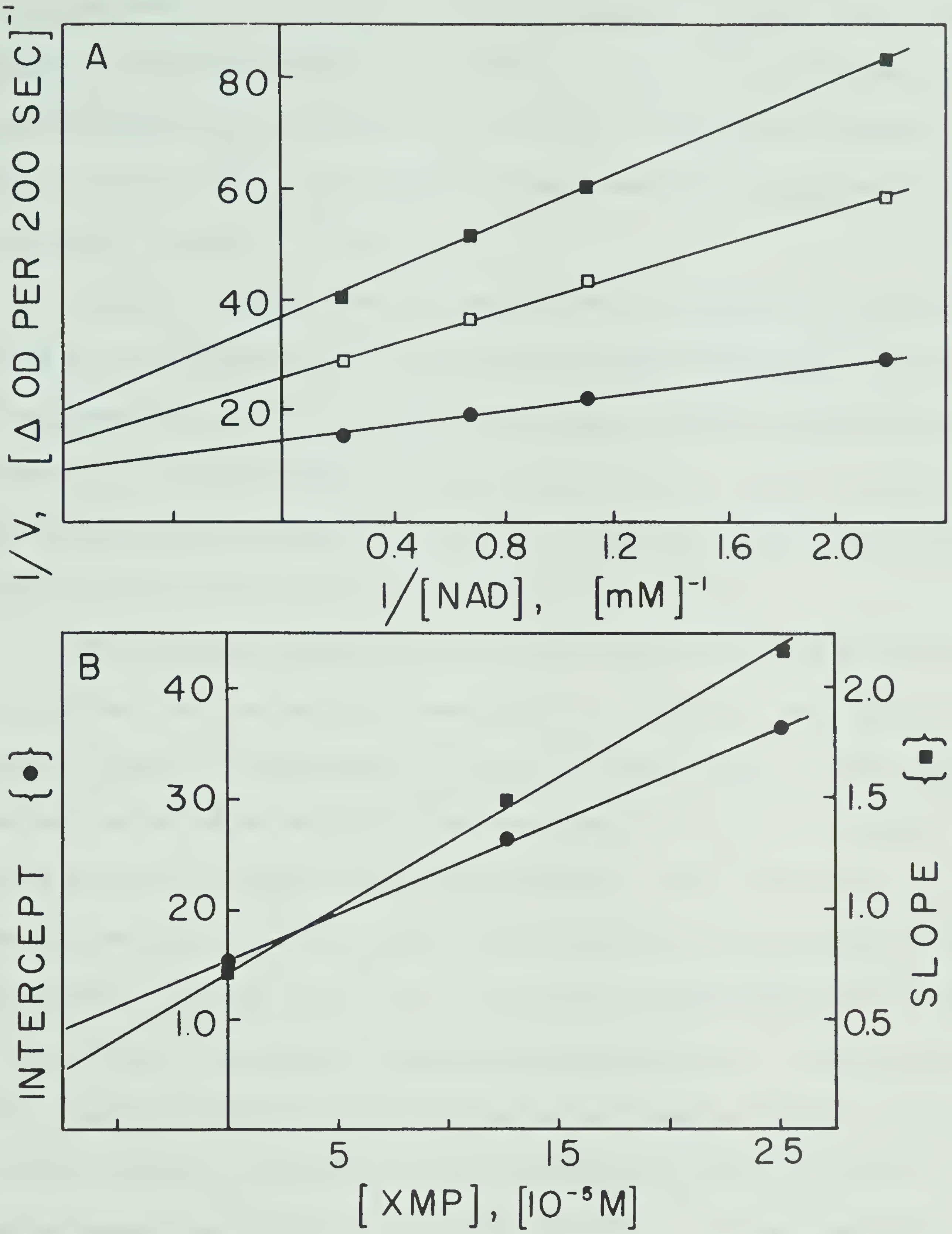


Figure 17: Frame A; reciprocal plot with NAD as the variable substrate and XMP as product inhibitor of IMP dehydrogenase at pH 6.0. IMP was 1.0 x 10^{-4} M. Frame B; secondary plots of slopes and intercepts against the XMP concentration.

[illegible]

[illegible]

[illegible]

(in parenthesis) are: relative maximal forward velocity, 0.03, (1.00); Michaelis constant for IMP, 4.2×10^{-5} M, (2.0×10^{-5} M); Michaelis constant for NAD, 0.2×10^{-3} M, (1.0×10^{-3} M); dissociation constant for IMP (K_{ia}), 19.5×10^{-5} M, (2.0×10^{-5} M) and dissociation constant for XMP (K_{iq}) 6.4×10^{-5} M, (12×10^{-5} M).

Since it appears that the kinetic model at pH 6.0 is similar to one at pH 8.1, the differences in the kinetic parameters may be attributed to ionizations on substrates, products or enzyme which are occurring over this pH range. In this region such ionizations may arise from the imidazole of histidine or the sulfydryl of cysteine on the enzyme, and from the phosphate of the nucleotides or the enolic group of XMP.

The decrease in maximum velocity at lower pH may be attributed to a decrease in either one or a combination of the k_5, k_7 and k_9 rate constants (Table IV). Decreases in k_5 and k_7 would reflect slower product release while slower catalysis would be due to a decreased k_9 rate constant. At pH 6.0, the dissociation constant for XMP is about one-third the value for IMP whereas at pH 8.1 it was 6-fold greater than the value for IMP. One might infer from this that the negatively charged XMP with an enolic pK of about 6 (57) dissociates faster from the enzyme than does the uncharged form, and that this is a major factor in the lower affinity of XMP relative to IMP at pH 8.1. Furthermore, this increased affinity at pH 6.0 of IMP dehydrogenase for XMP relative to IMP (18-fold) is almost certainly due in part to a decreased k_7 rate constant for the dissociation of XMP. This would account for at least some of the reduction in maximal velocity.

The much lower velocity concomitant with a two-fold increase in K_a suggests this pH change has caused a 60 to 70-fold reduction in k_1 which is the second order rate constant for the interaction of IMP and enzyme.

The paralleling 10 fold increase in the dissociation constant for IMP would necessitate a 6-7 fold decrease in k_2 which describes the first order dissociation of IMP from the enzyme. It is not possible at present to say whether these changes in k_1 and k_2 reflect the lesser affinity of the enzyme for the monovalent over divalent phosphate moiety of IMP or some change in the IMP binding site due to an enzymatic ionization. However, since the phosphate moiety of IMP is required for binding, the assumption that its state of ionization is important does not appear unwarranted. It would seem that at least part of the 60-70 fold decrease in k_1 may be attributed to the enzyme's inability to bind monovalent IMP as well as the divalent species. At pH 8.1 only 1.5% of the IMP species are monovalent whereas 67% are monovalent at pH 6.0 (pK 6.3).

V. IMP ANALOGUES AND IMP DEHYDROGENASE

1. Introduction

The general question as to how an enzyme and its substrate specifically interact prior to the actual catalysis has been much studied and speculated upon, but few really definitive answers have emerged. One method of approaching this problem has been to examine the inhibitory or substrate activity of a series of substrate analogues. By making small chemical modifications of the substrate or by attaching chemical groups of various sizes to different positions on the substrate, many workers have attempted to outline the possible bulk tolerances in a given enzyme-substrate system. The complete picture of which substrate positions are directly involved in the binding process can seldom if ever be accomplished by this type of structure-activity study. The overall electronic character of a molecule may be altered by even a slight structural modification, and one is always faced with assessing which change is producing the observed result. Although such a structure-activity study is beset with problems of interpretation, valuable knowledge on the mode of binding may often evolve.

Another means of studying binding is by observing the effects of pH on this process. By such a study one may be able to pinpoint substrate or enzymatic groups which are directly involved in binding as evidenced by the fact that the ionization state of such groups affects the extent of substrate binding (9). Unfortunately there are few meaningful studies of this type in the literature. In order to assess binding alone, the effects of pH on substrate or product dissociation constants are required,

as the effects on the Michaelis constants of the more complex kinetic models are very difficult to interpret. This then requires a complete initial velocity study at each pH value, and it must also be ascertained that the kinetic model describing the system, i.e., ordered or random, is not also changing as a function of pH. This entails much work which is probably a major reason why such a study is seldom undertaken.

Knowledge of bulk tolerances and tolerable substrate modification in any given system may be put to use in the design of active-site-directed irreversible inhibitors. This concept has been treated in much detail by Baker (28). The ability to design specific enzyme inhibitors is of much practical interest in the field of the various types of clinical chemotherapy.

With the aim of gathering information on the role played by the phosphate moiety of IMP in its binding to IMP dehydrogenase, various phosphate analogues of IMP were synthesized. Since inosine exhibits essentially no affinity for the IMP site of IMP dehydrogenase as evidenced by its lack of inhibition at high concentrations, it may be concluded that this phosphate group contributes to binding directly and/or can give rise to a particular molecular orientation which is more readily bound.

Earlier studies from this laboratory with IMP analogues in which a H, F, CH_3, or CH_2Cl group replaced one of the phosphate oxygens had shown these compounds were neither substrates, nor significantly strong inhibitors of the IMP dehydrogenase (31). Additional derivatives of IMP with nitrogen or sulfur replacing certain of the phosphate oxygens of IMP have been recently prepared (41), and their substrate activity with the bacterial IMP dehydrogenase will be described in this chapter.

2. Methods

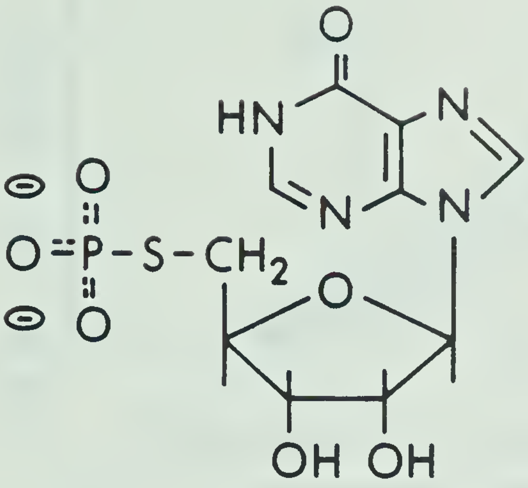
A highly purified phosphocellulose purified sample of IMP dehydrogenase was used with the same method of assay as described in Chapter IV.

The phosphate analogues of IMP shown in Figure 18, together with the names by which they will be referred, were synthesized in this laboratory. They were converted to the potassium salts by passage over a column of the potassium form of Dowex 50.

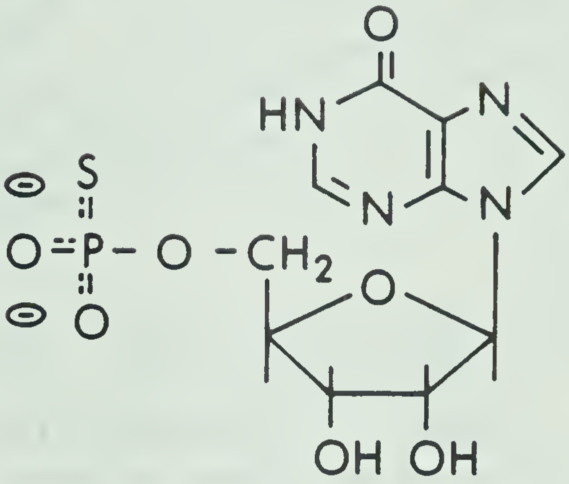
The secondary phosphate pK values for these IMP analogues were determined by titrating solutions of the potassium salts using a Corning Model 12 pH meter equipped with a standard (0-11 pH range), Thomas combination electrode. Standard HCl was added to 5 ml of a five to eight millimolar solution of the analogue in a 10 ml, pear-shaped, two-necked flask with a Kontes 2 ml microburet. Aqueous solutions were used for the titrations of the secondary phosphate ionizations of IMP, inosine 5'-phosphorothioate and 5'-thio-IMP, while the titration of the amide function of 5'-amino-IMP was done in the presence of 0.1 M KCl. Solutions were stirred continuously by means of a magnetic stirring device. The acid was standardized against tris-(hydroxymethyl)-aminomethane of primary standard grade.

3. Results

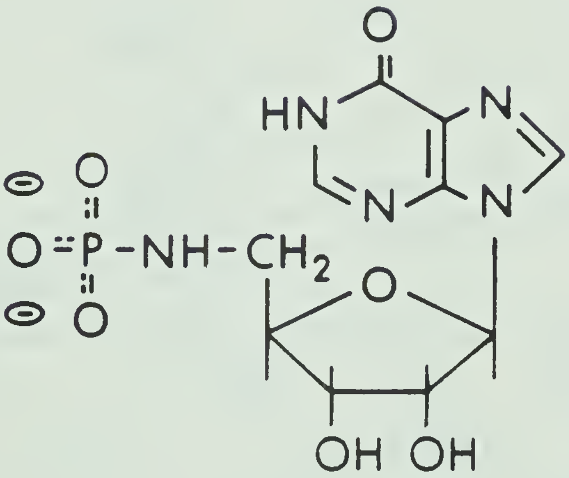
Initial experiments with the IMP analogues of Figure 18 indicated all were substrates of varying degree for IMP dehydrogenase. The initial velocity data for these compounds at pH 8.1 is shown in Figures 19, 20, and 21. Replots of all slopes and intercepts were linear. No product inhibition experiments were undertaken so the kinetic parameters listed in Table V were calculated utilizing the ordered Bi Bi equations of



5'-THIO-IMP



INOSINE 5'-PHOSPHOROTHIOAT



5'-AMINO-IMP

Figure 18: IMP analogues and the names by which they are referred to in the text.

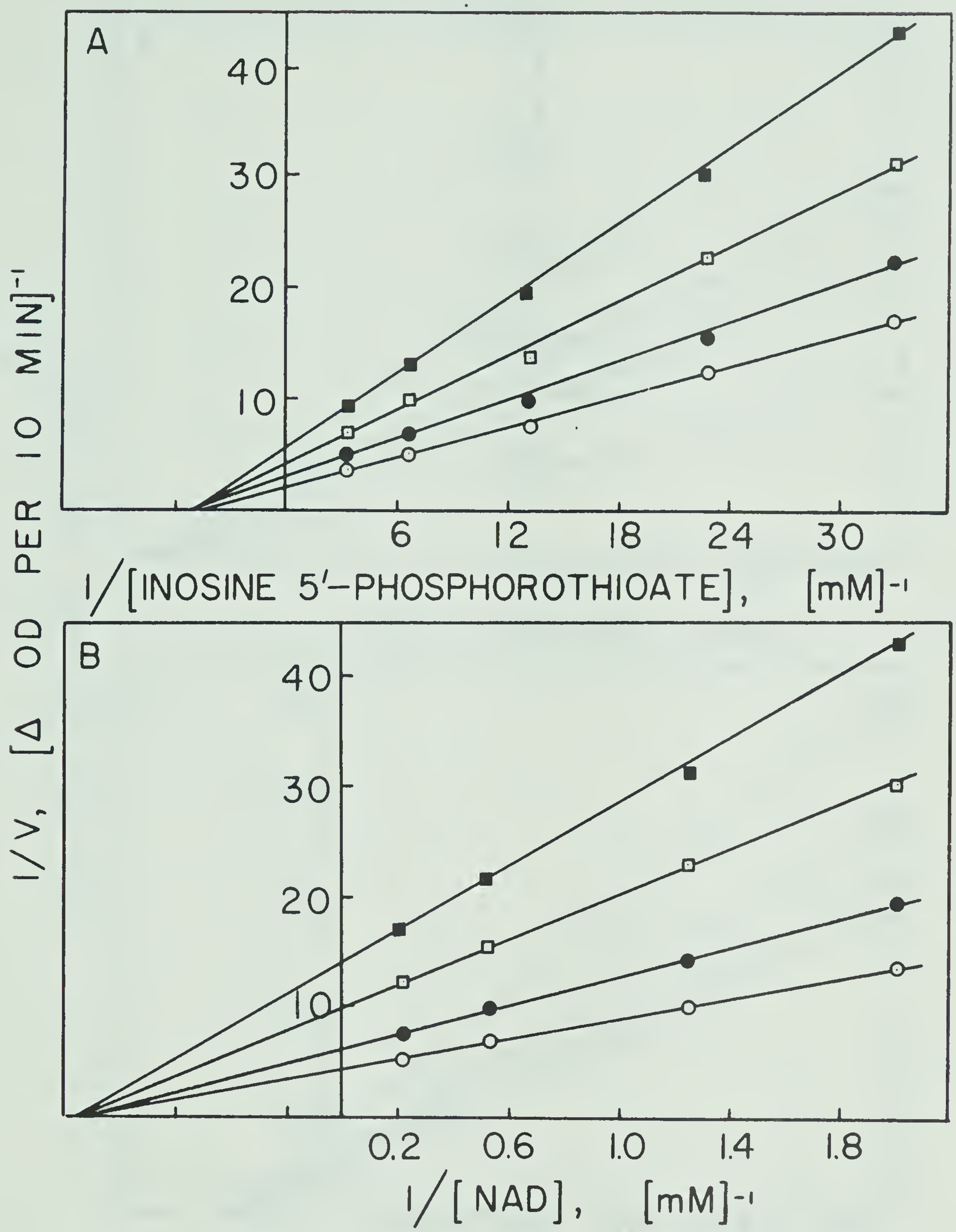


Figure 19: Initial velocity data for inosine 5'-phosphorothioate and IMP dehydrogenase at pH 8.1. Frame A; inosine 5'-phosphorothioate varied at fixed NAD concentrations of 4.7 x 10^{-4} M (■), 7.9 x 10^{-4} M (□), 1.9 x 10^{-3} M (●), and 4.7 x 10^{-3} M (○). Frame B; NAD varied at fixed inosine 5'-phosphorothioate concentrations of 3.0 x 10^{-5} M (■), 4.5 x 10^{-5} M (□), 7.5 x 10^{-5} M (●), and 3.0 x 10^{-4} M (○). OD changes measured at 290 mμ.

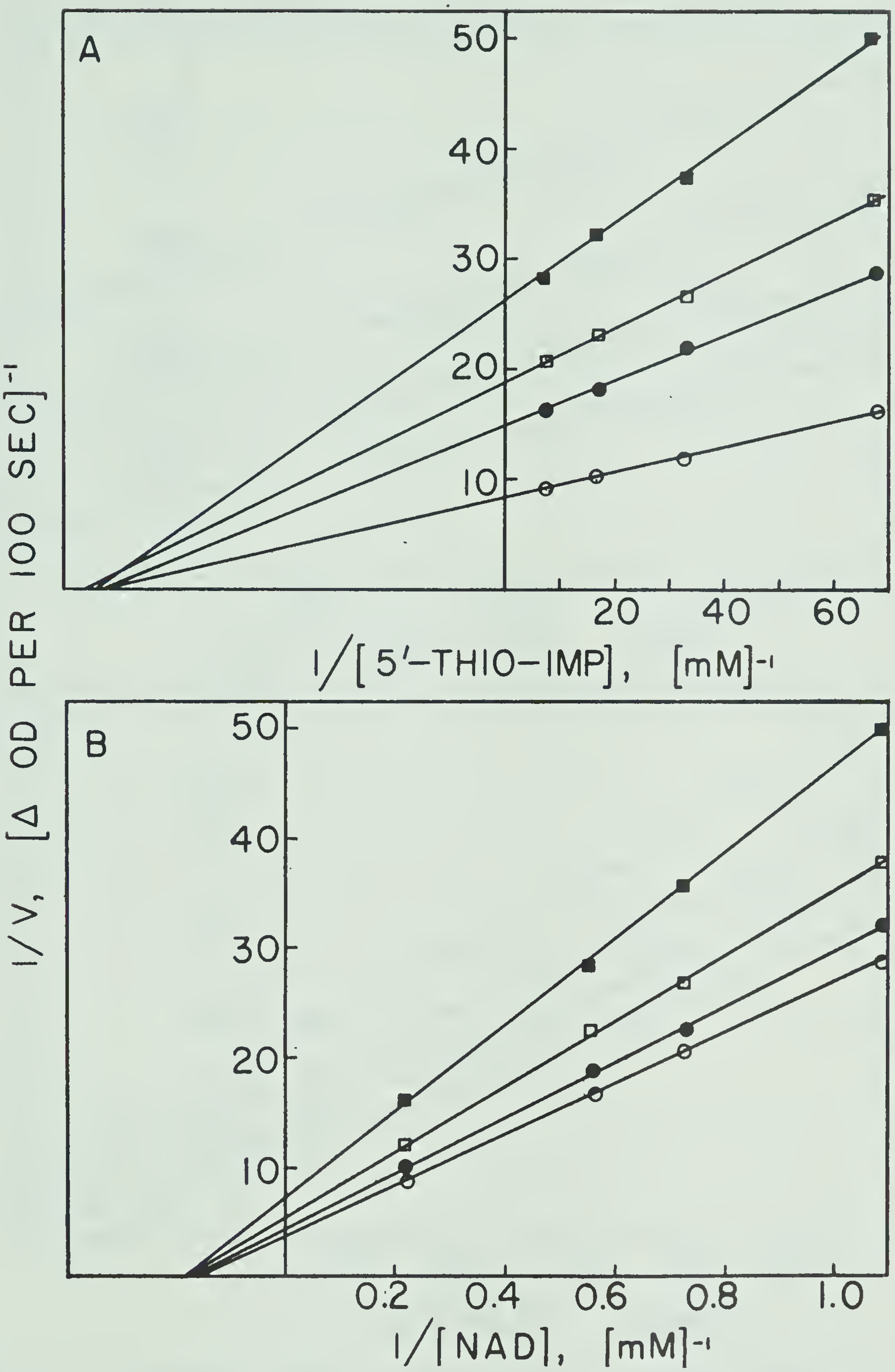


Figure 20: Initial velocity data for 5'-thio-IMP and IMP dehydrogenase at pH 8.1. Frame A; 5'-thio-IMP varied at fixed NAD concentrations of 9.2×10^{-4} M (■), 1.4×10^{-3} M (□), 1.8×10^{-3} M (●), and 4.6×10^{-3} M (○). Frame B; NAD varied at fixed 5'-thio-IMP concentrations of 1.5×10^{-5} M (■), 3.0×10^{-5} M (□), 6.0×10^{-5} M (●), and 1.2×10^{-4} M (○). OD changes measured at 290 mμ.

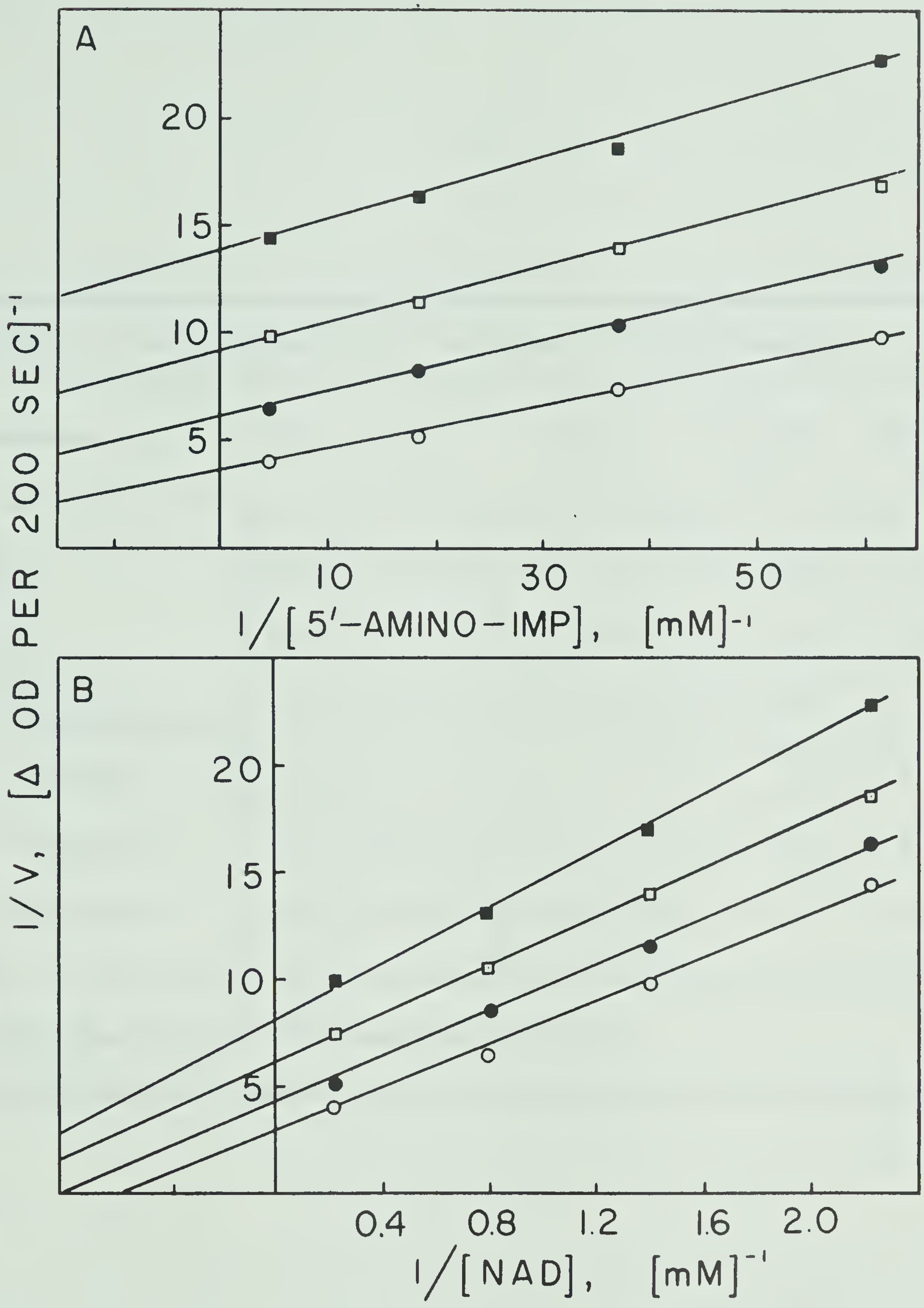


Figure 21: Initial velocity data for 5'-amino-IMP and IMP dehydrogenase at pH 8.1. Frame A; 5'-amino-IMP varied at fixed NAD concentrations of 4.5×10^{-4} M (■), 7.2×10^{-4} M (□), 1.3×10^{-3} M (●), and 4.5×10^{-3} M (○). Frame B; NAD varied at fixed 5'-amino-IMP concentrations of 1.6×10^{-5} M (■), 2.7×10^{-5} M (□), 5.0×10^{-5} M (●), and 2.2×10^{-4} M (○). OD changes measured at 340 mμ.

[illegible]

[illegible]

TABLE V: Kinetic Parameters for Substrates of IMP Dehydrogenase.

Analogue	pH	K_{IMP} M	K_{NAD} M	$K_{(i)\ IMP}$ M	Relative V_{max}
IMP	8.1	2.0×10^{-5}	1.0×10^{-3}	2.0×10^{-5}	1.00
IMP	6.0	4.2×10^{-5}	0.2×10^{-3}	19.5×10^{-3}	0.03
Inosine 5'-Phosphorothioate	8.1	21×10^{-5}	0.9×10^{-3}	23×10^{-5}	1.05
5'-thio-IMP	8.1	1.3×10^{-5}	5.3×10^{-3}	1.7×10^{-5} 1.9×10^{-5} [a]	0.75
5'-amino-IMP	8.1	3.8×10^{-5}	2.0×10^{-3}	0.5×10^{-5}	0.67
5'-amino-IMP	6.0	7.4×10^{-5}	0.4×10^{-3}	13×10^{-5}	0.01

[a] Determined by inhibition experiments with IMP.

Chapter IV. The corresponding IMP values are included for comparison.

The reduced maximal velocity and higher Michaelis constant for NAD with 5'-thio-IMP resulted in little of this analogue being oxidized under the normal assay conditions employed with IMP as the substrate. Hence, it was possible to use this analogue as an inhibitor of the enzyme catalyzed oxidation of IMP. Figure 22 shows that when treated in this manner the 5'-thio-IMP gave linear competitive inhibition as the IMP concentration was varied. The dissociation constant of 1.9×10^{-5} M for this analogue as determined from the horizontal intercept of the replot is in good agreement with the value of 1.7×10^{-5} M obtained from initial velocity data (Table V). This consistency is additional evidence that the ordered Bi Bi kinetic model is a satisfactory working model for the bacterial IMP dehydrogenase. The initial velocity data for 5'-amino-IMP at pH 6.0 is shown in Figure 23.

The titration curves for the secondary phosphate groups of IMP, inosine 5'-phosphorothioate, and 5'-thio-IMP, together with the curve for the ionization of the amide moiety of 5'-amino-IMP are shown in Figure 24. The pK values as determined directly from these curves for the above compounds are 6.3, 5.2, 5.7 and 8.5, respectively. The acid lability of 5'-amino-IMP prevented determination of the pK of the secondary phosphate.

As with IMP, the equilibrium for the oxidation of these analogues by IMP dehydrogenase is very much to the right, since with excess NAD, the final optical density change at 340 mμ was greater than 95% theoretical as calculated spectrophotometrically using the extinction coefficient of IMP for all the IMP analogues.

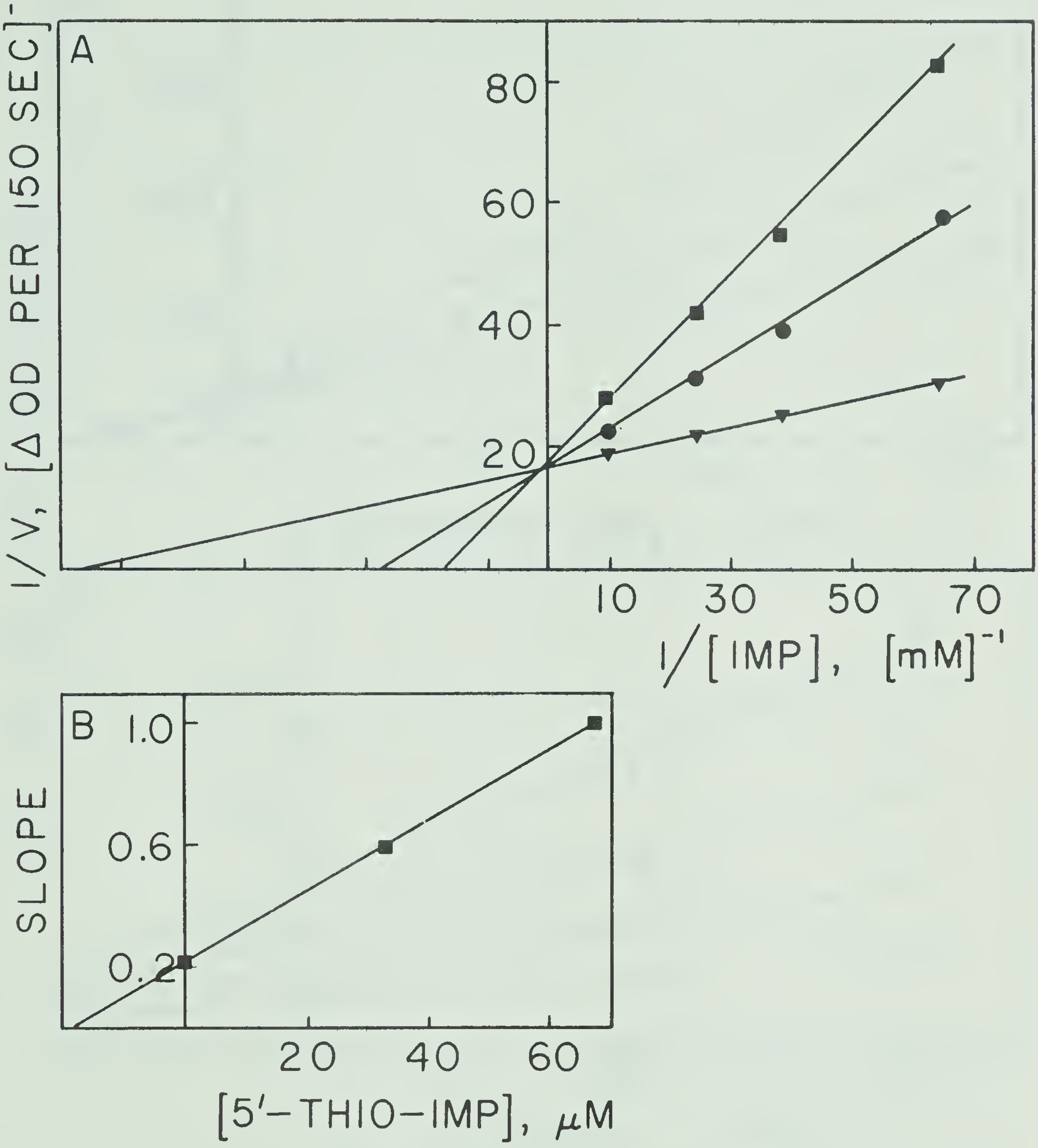


Figure 22: Inhibition of IMP dehydrogenase by 5'-thio-IMP. Frame A; IMP varied with 5'-thio-IMP concentrations of 0 (▽), 3.3 x 10^{-5} M (●), and 6.7 x 10^{-5} M (■). The NAD concentration was fixed at 1 x 10^{-3} M. Frame B; replot of slopes vs. 5'-thio-IMP concentration.

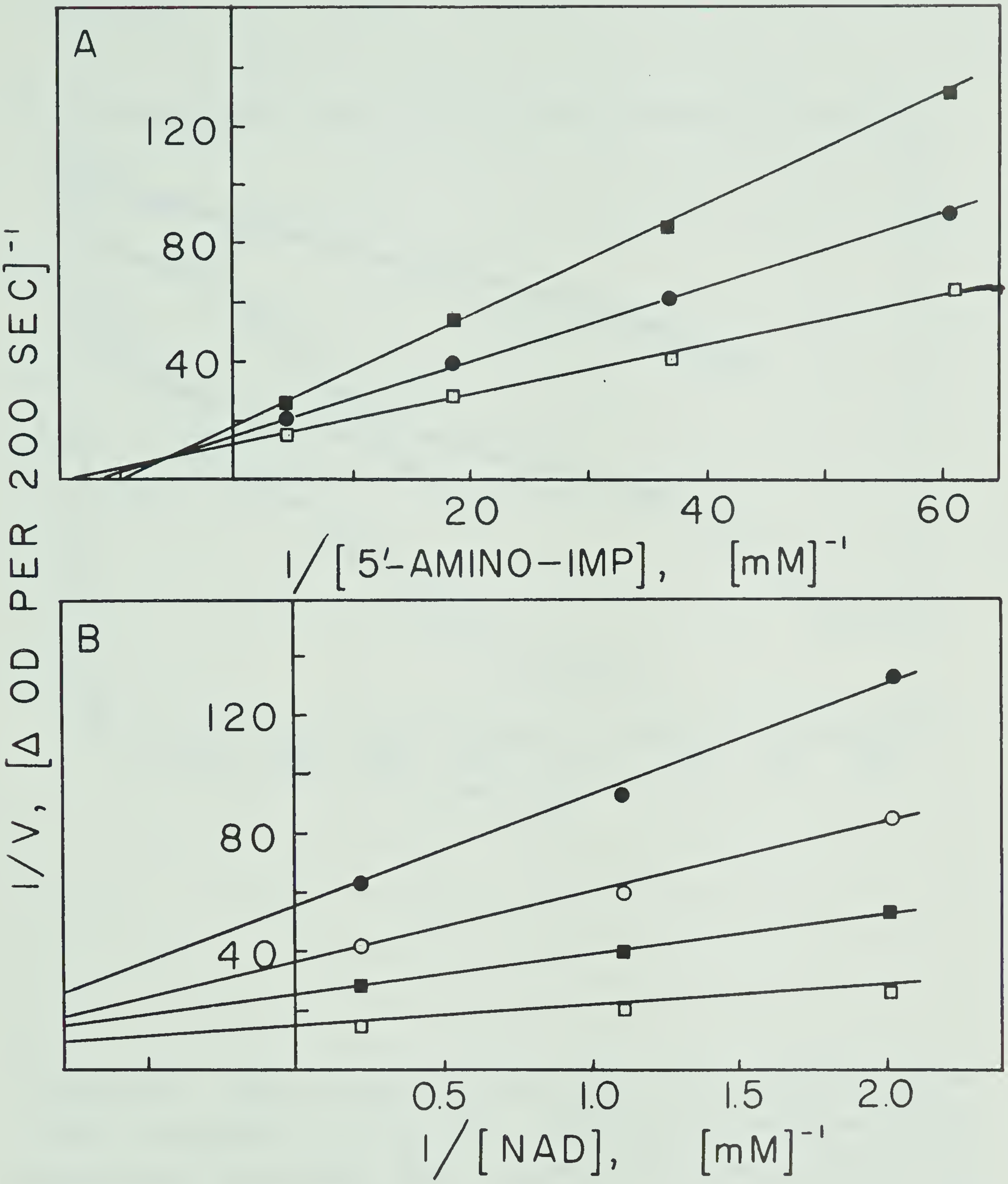


Figure 23: Initial velocity data for 5'-amino-IMP and IMP dehydrogenase at pH 6.0. Frame A; 5'-amino-IMP varied at fixed NAD concentrations of 4.5×10^{-4} M (■), 1.1×10^{-3} M (●), and 4.5×10^{-3} M (□). Frame B; NAD varied at fixed 5'-amino-IMP concentrations of 1.6×10^{-5} M (●), 2.7×10^{-5} M (○), 5.5×10^{-5} M (■), and 2.2×10^{-4} M (□). OD changes measured at 340 mμ.

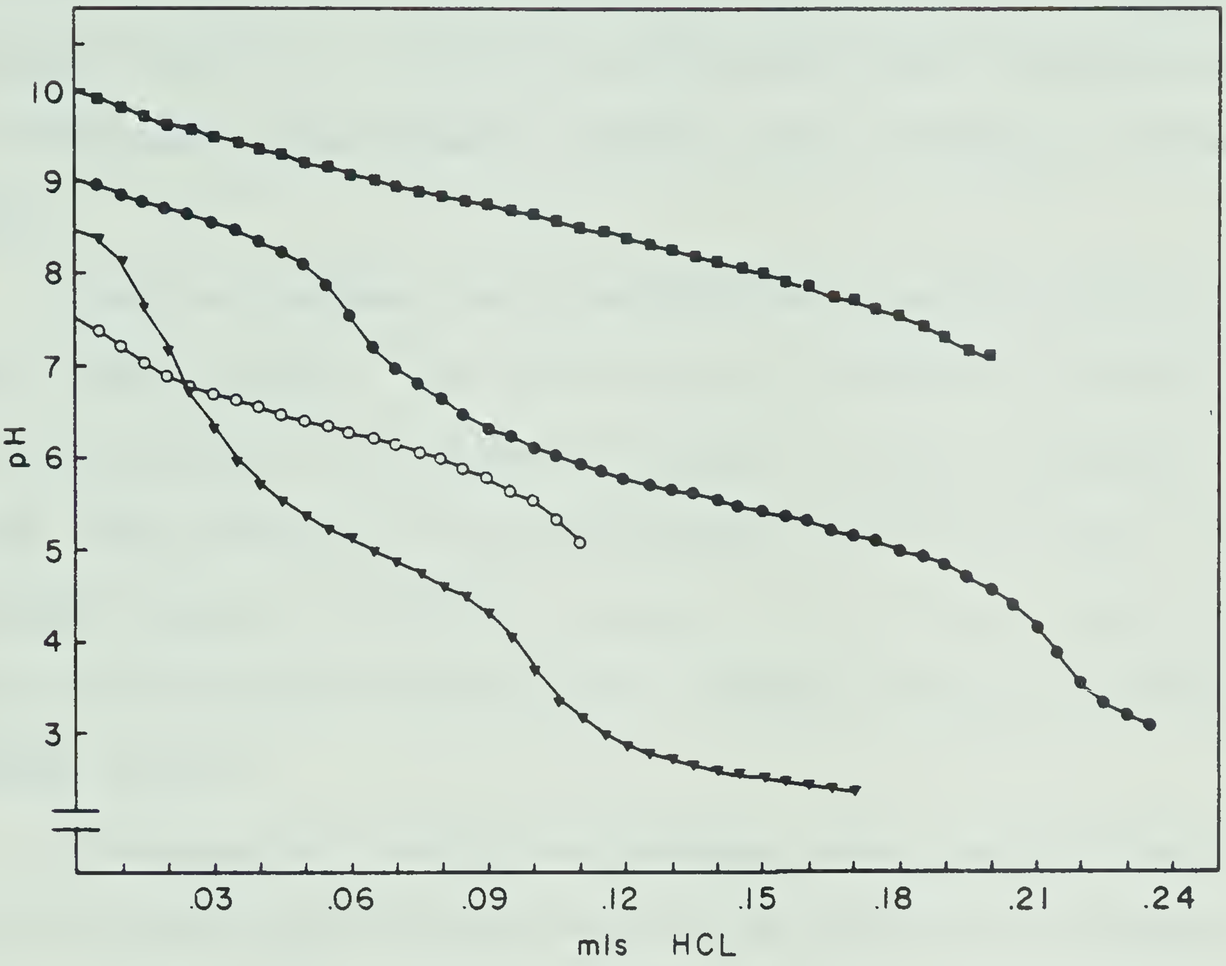


Figure 24: The titration curves for the secondary phosphate ionization of IMP (O), inosine 5'-phosphorothioate (▽), and 5'-thio-IMP (●); and for the amide moiety of 5'-amino-IMP (■). The HCl concentration was 0.27 M for the 5'-amino-IMP and 5'-thio-IMP titrations and 0.54 M for the IMP and inosine 5'-phosphorothioate titrations.

[illegible]

4. Discussion

The IMP analogues of Figure 18 are more similar to IMP from both a structural and electronic viewpoint than were the earlier phosphate analogues tested (2). This work and the earlier work both demonstrate the involvement of the phosphate moiety of IMP in binding to the dehydrogenase.

There are, perhaps, two roles which the phosphate group may play in the binding process. The most obvious is the direct contribution due to the interaction of the oxygen or phosphorus groups with groups on the enzyme. Baker (66) has listed approximately 20 possible ways in which a phosphate group may conceivably interact with its enzyme binding site through combinations of electrostatic, hydrogen bonding, and charge-transfer effects.

The second way in which the phosphate moiety could influence binding is by conferring on the IMP molecule the particular configuration which is recognized by the enzyme. Whether the nucleosides and nucleotides exist in solution in the _syn_ or _anti_ conformation has been of much current interest (67, 68, 69, 70), but the matter has not been fully resolved. A recent NMR study of aqueous nucleotide solutions shows the natural purine nucleotides are probably in the _anti_ conformation (52). This is not too surprising when molecular models are examined. CPK atomic models (Ealing Corporation) show that rotation into the _syn_ conformation is subject to various steric hindrances particularly with the C-2 substituted purine nucleotides such as GMP and XMP. One rather unexpected result which did emerge from this NMR study was the interaction between the phosphate field and the C-8 proton field. This shows that the phosphate group or at least part of its hydration shell is in close

proximity to this C-8 proton. Molecular models show that the phosphate group possesses considerable freedom of rotation around the 5'-carbon of the ribose ring. It is thus not immediately apparent why this group prefers to be oriented above the plane of the ribose ring and close to C-8 of the purine. The specificity of IMP dehydrogenase for IMP but not inosine might then eventually be explained if it was found that the nucleosides existed predominately in the syn conformation while the nucleotides were in the anti form. It must always be remembered, though, that syn and anti are the extreme opposites and any intermediate conformation might also exist.

The present results with the IMP analogues may be examined with respect to the rate constant expressions shown in Table IV of Chapter IV. The critical assumption here is that an ordered Bi Bi kinetic model is indeed a suitable choice for this IMP dehydrogenase system.

When inosine 5'-phosphorothioate replaces IMP as a substrate, there is no significant change in V_{max} or Michaelis constant for NAD (Table V). One infers from the expressions of Table IV that there have been then no great changes in the rates of catalysis or the release of the products which are described by the k_9, k_5 and k_7 rate constants. Since K_a is equal to $V_{max}/k_1 E_t$, it follows that k_1 must have decreased by a factor of ten to give rise to the observed ten-fold increase in Michaelis constant if the V_{max} remains unchanged. A decrease of this magnitude in k_1 would also be expected to give rise to a ten-fold increase in K_{ia}. This in fact was observed. The results with this analogue may then be explained by a single change in the rate constant which describes the rate at which the analogue and enzyme interact. Such a decrease may be due to a difference in conformation of the nucleotide

and/or to a difference in binding energy. In the latter case one might also expect a change in the rate of dissociation of analogue from the enzyme. As this does not appear to be the case, it could be concluded that introduction of the sulfur atom into this position is causing a slight difference in IMP conformation which slows the binding process. If IMP is bound with the phosphate group rotated up near the purine C-8 (52), it might be expected that introduction of the larger sulfur for one of the oxygen atoms could result in a different preferred conformation which was not bound as readily.

The 25% reduction in V_{max} with the 5'-thio-IMP appears to account for the slight decrease in Michaelis constant for this analogue. The unaltered dissociation constant for the analogue as compared to IMP suggests the k_1 and k_2 constants may be essentially the same as for the IMP case. This may be taken as an indication that the bridge oxygen of IMP does not significantly contribute to total binding energy. One might have postulated that this oxygen may act as an electron donor in hydrogen bond formation. This now seems unlikely as the sulfur analogue binds as well as IMP, but the electron pair of sulfur enters into such bonding much less readily than as in the case of oxygen. The cause of the five-fold increase in the Michaelis constant for NAD remains unexplained, but the expressions in Table IV suggest that this change most likely reflects a decreased rate constant (k_3) for the addition of NAD to the enzyme-IMP complex.

The decreased maximal velocity coupled with a two-fold increase in Michaelis constant for the 5'-amino-IMP derivative would require a three-fold reduction in the k_1 rate constant. To obtain the observed smaller dissociation constant with such a reduced k_1 value further re-

quires that the k_2 constant be reduced by a factor of six. Why this analogue should be released more slowly than IMP is unclear and speculation is complicated as both the charged and uncharged amide moieties are present at this pH. The essentially complete protonation of the amide function of this 5'-amino-IMP at pH 6.0 does not appear to give any more information as the results are still qualitatively the same.

An increase in the Michaelis constant for NAD is observed when the bridge oxygen is replaced by nitrogen. This is similar to the case for the sulfur substitution. How the group at this position can effect a change in what is presumably the rate at which NAD adds to the enzyme is unknown. One is left with the contemporary explanation of many not understood phenomenon of protein chemistry, namely, some conformational change in protein structure has resulted from the binding of these modified analogues which has consequently altered the NAD site. If it were assumed that IMP was bound in the syn conformation, it would be easier to visualize how modifications in the phosphate region of the molecule might influence the NAD binding as the phosphate group, the purine C-2 group(s) and the nicotinamide portion of NAD could be in close proximity. However, as both the NMR studies (52) and the molecular models suggest that XMP probably exists in the anti form, it is more reasonable to presume that this too is the form bound by IMP dehydrogenase.

VI. INACTIVATION OF IMP DEHYDROGENASE BY 6-CHLOROPURINE 5'-PHOSPHATE

1. Introduction

It has been reported that a structural analogue of IMP, 6-chloro-9-β-D-ribofuranosylpurine 5'-phosphate, will progressively inactivate the IMP dehydrogenase of *A. aerogenes* (20, 23) and Erlich acites cells (24). The progressive nature of the inhibition suggested that covalent bond formation may be involved in this inactivation. Such an inactivation was of interest in that some information may be obtained as to the necessary and reactive enzymic groups. This inhibition was of further interest as 6-chloropurine has been reported to inhibit growth in certain mouse tumors (26), and it has also been used clinically in treating acute leukemia in humans (25). The 6-chloropurine also inhibits *de novo* synthesis of guanine nucleotides in mouse Erlich acites cells (71, 72). As 6-chloropurine may be converted to its 5'-phosphate *in vivo* (73), it was of interest to further characterize the forementioned inactivation, as IMP dehydrogenase may be the site of action of this purine analogue.

The inactivation by 6-chloropurine nucleotide was slowed by IMP and GMP which is a competitive inhibitor of IMP (20, 23). With enzyme preparations exhibiting an absolute thiol requirement for activity, there was a similar requirement for inactivation to occur (20). The inhibition, once established, could not be reversed by IMP or dialysis. The 6-chloropurine nucleoside was about 200 times less effective than the 5'-phosphate for inactivating IMP dehydrogenase (23). These results were taken to suggest that this inhibitor was reacting covalently at the IMP binding site (23).

If 6-chloropurine nucleotide was reacting covalently with the enzyme, the reaction would most likely involve the relatively electron deficient C-6 position of the purine ring. One may envisage the nucleophilic attack on this position by some reactive enzymic group with the concomitant release of chloride ion. As purines have very distinctive spectra in the near ultraviolet region, it was thought that covalent bond formation between enzyme and inhibitor may cause a spectral shift, and it may be possible to identify the enzymic group from this shift. Such experiments are described in this chapter.

Once it was established that covalent bond formation was involved, it was of interest as to how the inhibitor might interact with the enzyme. The steric similarity of 6-chloropurine nucleotide to IMP, together with the protection data, indicated the inhibitor may first bind to the IMP dehydrogenase to give a Michaelis-type complex, and that in this complex the C-6 of the purine ring might be attacked by a neighboring enzymic group. The inhibitor could then not dissociate and would block the site for any future IMP binding. The rate of the 6-chloropurine nucleotide inactivation was studied in an attempt to determine whether this inactivation proceeded through an enzyme-inhibitor complex, or whether a random bimolecular process was involved.

A new synthesis of 6-chloropurine nucleotide is described. This procedure gives good yields and is somewhat simpler than the previously described method (74).

2. Synthesis of 6-Chloro-9-B-D-ribofuranosylpurine 5'-Phosphate

The procedure was based on that developed by Robins (75) for the conversion of 6-thioinosine to 6-chloropurine ribonucleoside. A suspen-

sion of 50 mg of barium 6-thioinosinate (76) in 2 ml of methanol was cooled to below -10° in an acetone-solid CO_2 bath with exclusion of moisture. Chlorine gas was bubbled slowly into the mixture for 15 minutes during which the temperature was maintained below -5°. The suspended material dissolved within 7 minutes. The yellow solution was kept for an additional 15 minutes at the same temperature, after which cooling was continued and nitrogen was bubbled through to remove the excess of chlorine. Saturated methanolic ammonia, precooled to -10°, was added to adjust the pH of the solution to 7. A heavy white precipitate formed; water was added to dissolve it (final volume of the mixture was 8 ml); and 0.5 ml of 1 M barium acetate was added. After 2 hours at 4° the solution was centrifuged to remove small amounts of insoluble material. Ethanol (32 ml) was added and the mixture was stored at 4° overnight. The white precipitate was collected by centrifugation and washed once with 8 ml of aqueous 80% ethanol. The product was dried over NaOH *in vacuo*, then dissolved in 2 ml of water, and applied to a column containing about 20 equivalents of Dowex 50 (Na^+) ion-exchange resin. The column was washed with water and 10 ml of effluent was collected. The spectral characteristics of this solution in 0.05 M acetate buffer (pH 4.8) were λ_{max} 264 mμ, λ_{min} 226.5 mμ; absorbancy ratio $A_{264} : A_{226.5} = 4.3$, $A_{250} : A_{260} = 0.76$, and $A_{280} : A_{260} = 0.19$. The values reported for 6-chloro-9-β-D-ribofuranosylpurine 5'-phosphate by Hampton and Maguire (76) are 263 and 226 mμ and absorbancy ratios 4.0, 0.82 and 0.175, respectively. Chromatography of the solution in 1-butanol-acetic acid-water (5:3:2), isopropyl alcohol-1% $(NH_4)_2SO_4$ (2:1), and isoamyl alcohol-0.3 M phosphate (pH 6.9) (1:1) showed one fluorescent spot with the same R_F as 6-chloropurine nucleotide (76). The yield of 6-chloropurine nucleotide by the present procedure was determined spectro-

photometrically to be 92%. The barium salt of 6-chloropurine nucleotide was precipitated from the solution of the sodium salt with barium acetate and ethanol in the usual manner. When dried over P_2O_5 for 3 hours at 0.1 mm and 100^o it showed λ_{max} 264 mμ (ε 8260) at pH 4.8 (reported previously, ε 8400 at 263 mμ); this extinction coefficient corresponds to the anhydrous form of the barium salt of this nucleotide, and this form is known to be produced by the above drying conditions (76).

3. Spectral Study

a. Methods and Results

To a phosphocellulose purified enzyme fraction was added a solution of saturated $(NH_4)_2SO_4$ to give a final salt concentration of 65%. After standing overnight at 4^o, the precipitate was collected by centrifugation at 14,000 rpm for 15 minutes in a Servall R-2. The precipitate was dissolved in cold 0.02 M potassium phosphate buffer, pH 7.4, which contained glutathione (2×10^{-3} M) and KCl (0.1 M). The protein concentration was adjusted to 2 mg per ml as judged by the optical density ratio at 260 and 280 mμ. At various times, three 5 μl portions of a neutral 3.3×10^{-3} M solution of 6-chloropurine nucleotide solution were added to 1 ml of the above enzyme solution. Spectra were taken at different time intervals on a Cary Model 15 spectrophotometer using the untreated protein solution as a blank. The cell compartments were thermostated at 23^o.

Figure 25-A shows the spectra at various time intervals after the first 5 μl addition of 6-chloropurine nucleotide. The peak at 263 mμ which is characteristic of 6-chloropurine nucleotide decreases with time as a new peak in the region of 290 mμ appears. An isobestic point was apparent at 272 mμ. The second 5 μl portion of 6-chloropurine nucleotide

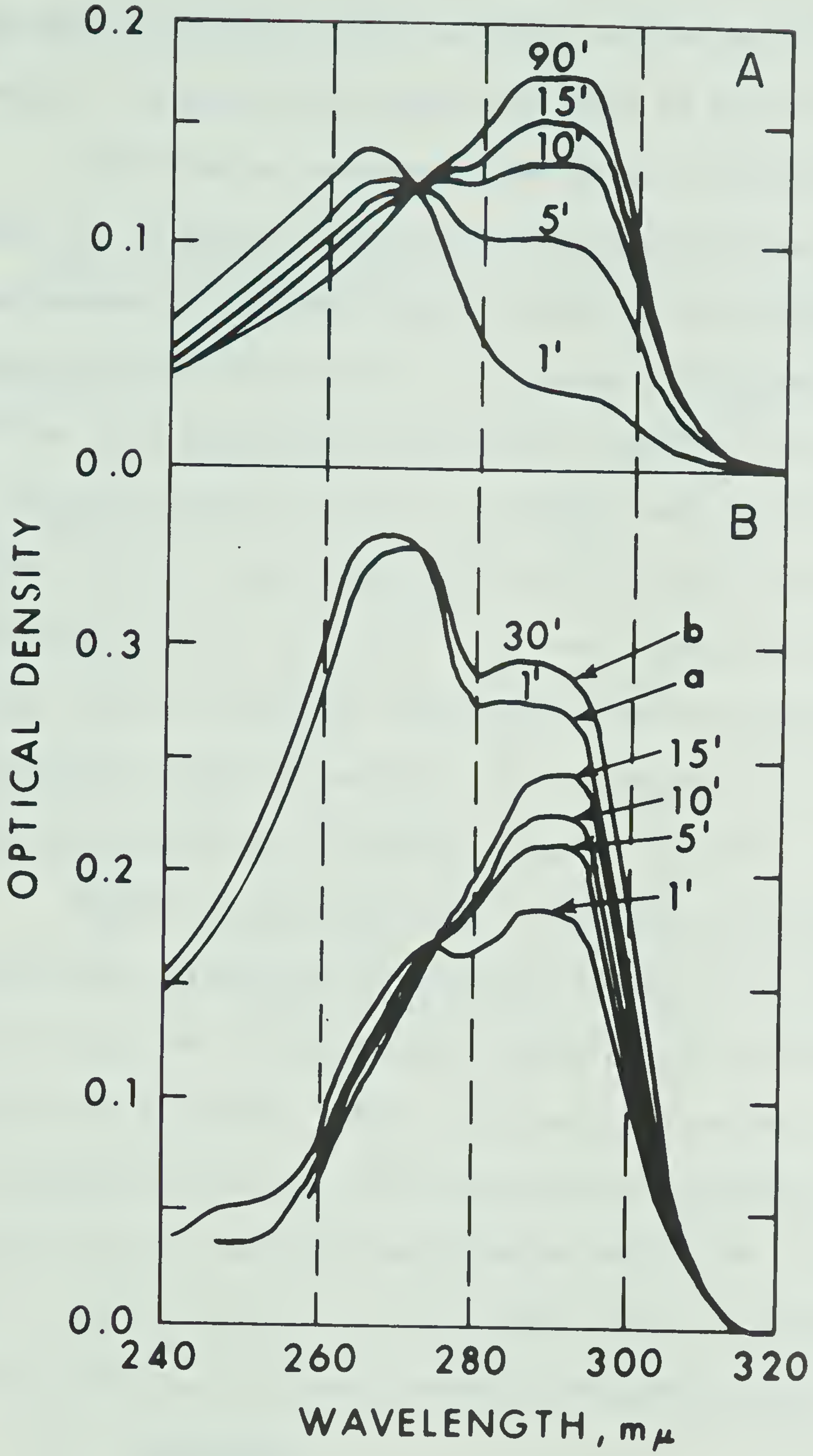


Figure 25: Spectral changes resulting from reaction of 6-chloropurine nucleotide with IMP dehydrogenase. The 6-chloropurine nucleotide (16.5 mμmoles) was added to ca. 10 mμmoles of the enzyme under conditions described in the text (Frame A); a further 16.5 mμmoles of 6-chloropurine nucleotide was added after 100'(Frame B) and after another 20 minutes (Frame B, curves a and b).

was added 95 minutes after the first and the third 20 minutes after the second. The resulting spectra are shown in Figure 25-B.

There was no increase at 290 mμ if 6-chloropurine nucleotide was added to the buffer solution containing glutathione and KCl but no protein. The increase in optical density at 290 mμ was accompanied by a decrease in enzyme activity (Figure 26). Replacing 6-chloropurine nucleotide (16.5×10^{-6} M) by 6-chloropurine nucleoside (2.0×10^{-4} M) resulted in no change in optical density at 290 mμ in 2 hours, and there was also no loss of enzyme activity. When IMP (2.0×10^{-4} M) was included with the chloropurine nucleotide (16.5×10^{-6} M), the increase in optical density at 290 mμ was 0.03 with 18% inactivation after 20 minutes and 0.12 with 50% inactivation after 90 minutes. In the absence of IMP, 50% inactivation occurred after 10 to 15 minutes (Figures 25, 26).

Figure 27 shows the rate of increase in the optical density at 290 mμ when glutathione or β-mercaptoethanol react non-enzymatically at pH 9.0 with the 6-chloropurine nucleotide and nucleoside. After sufficiently long reaction times, the absorption maximum at 263 mμ of the 6-chloropurine nucleoside was replaced by a new maximum at 292 mμ characteristic of a 6-(alkylmercapto)purine nucleoside at slightly alkaline pH (77). The nucleoside reacted 5 times faster than nucleotide in bicarbonate buffer and 8.5 times faster in Tris-HCl buffer.

b. Discussion

The time dependent spectral shift from 263 mμ to 290 mμ is consistent with the nucleophillic attack by the sulfydryl group of a cysteine residue on the purine C-6 of 6-chloropurine nucleotide to give a 6-(enzyme-mercapto)purine nucleotide derivative. This 290 mμ maximum is very characteristic of the 6-(alkylmercapto)purines (77, 78). Nucleo-

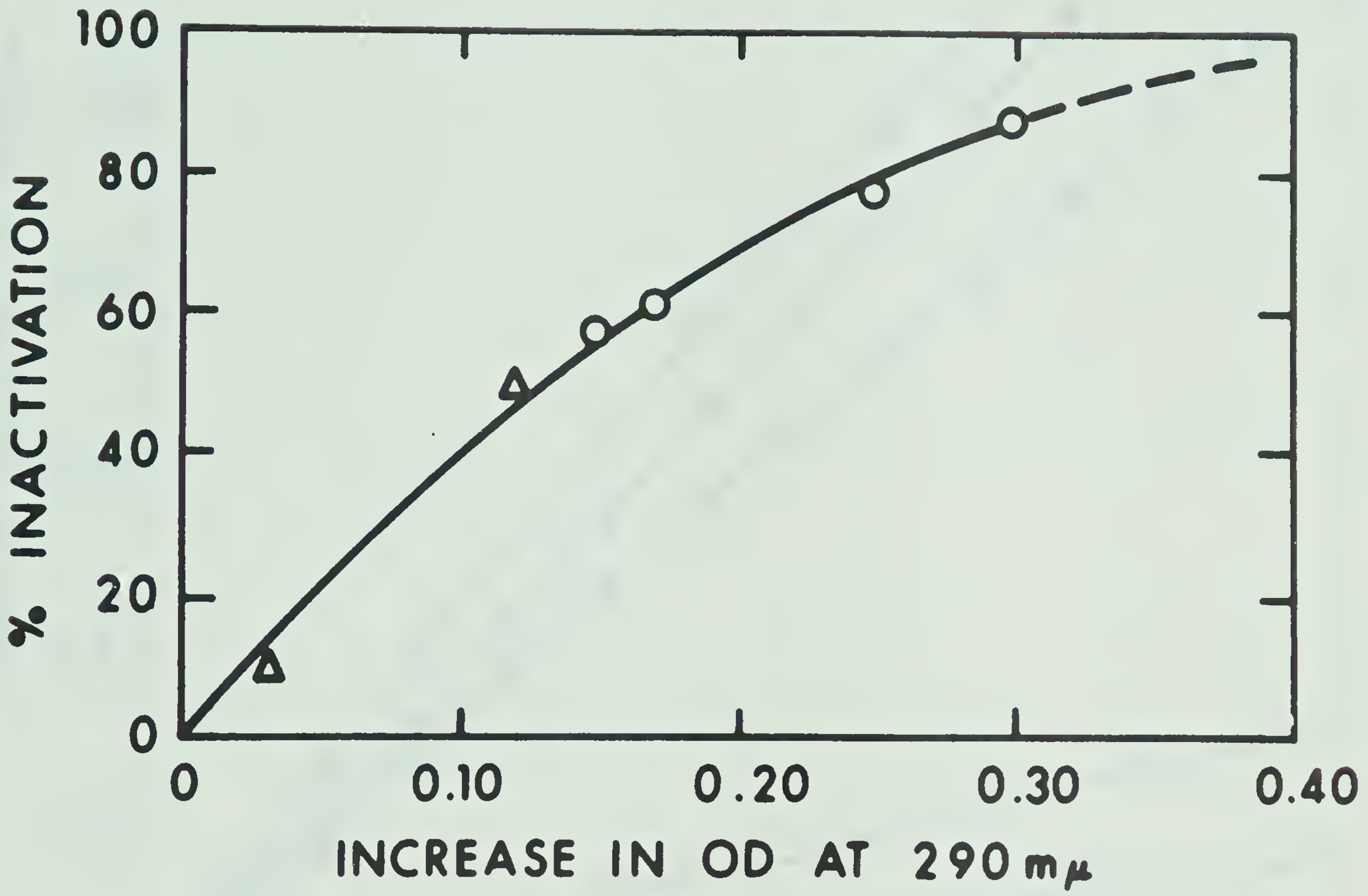


Figure 26: Inactivation of IMP dehydrogenase by 6-chloropurine nucleotide as a function of the increase in OD at 290 mμ. Values presented as open circles are from the experiment of Figure 25 and values presented as open triangles are from a parallel experiment carried out in the presence of 200 μM IMP and (initially) 16.5 μM 6-chloropurine nucleotide.

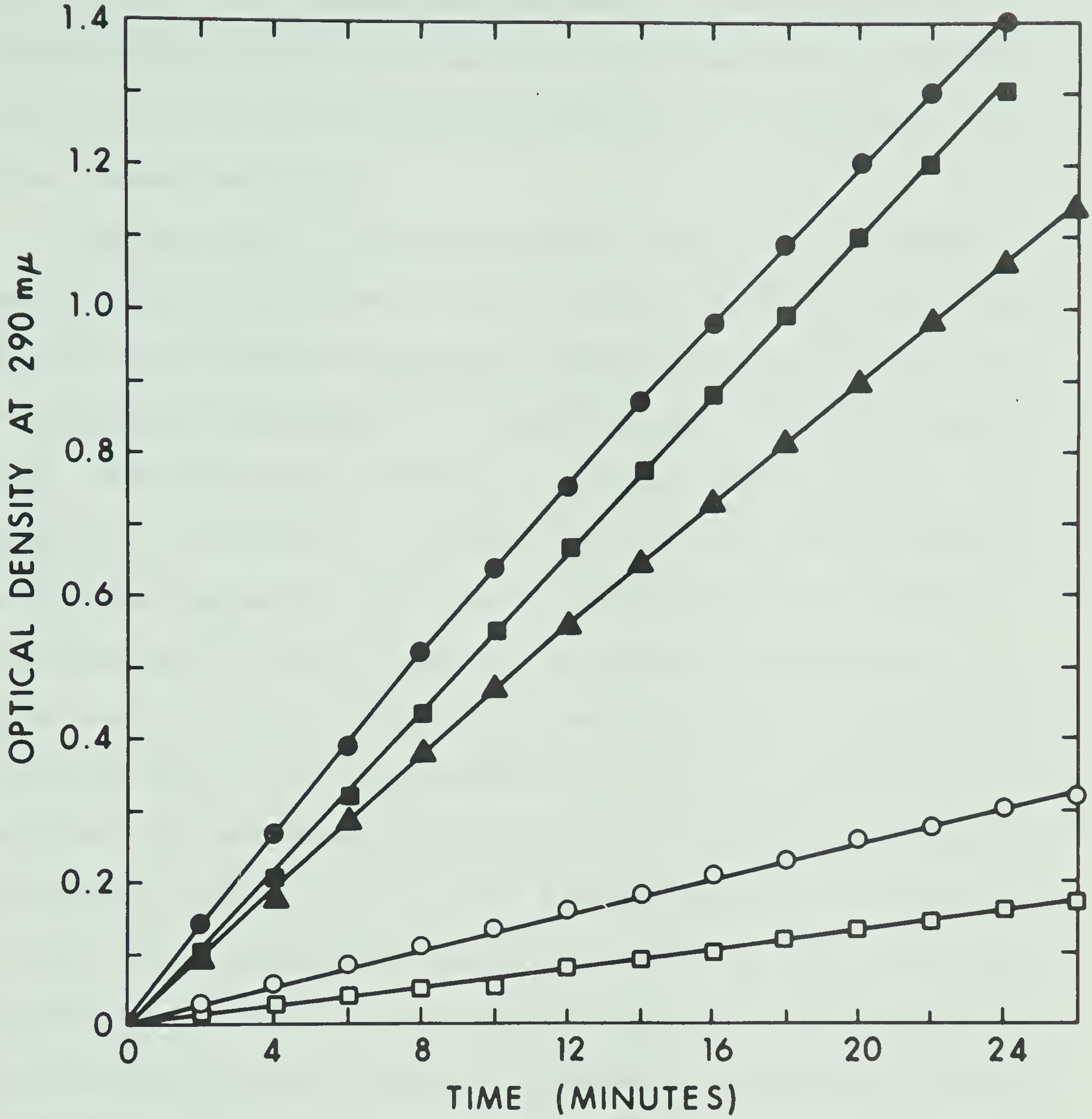


Figure 27: Rates of nucleophillic attack on carbon-6 of 6-chloropurine nucleotide and of 6-chloropurine nucleoside by aliphatic mercaptans. All reactants 1 mM and buffers 0.1 M and pH 9.0; temperature, 24°. Nucleoside and GSH in sodium bicarbonate buffer (●); nucleotide and GSH in bicarbonate (○); nucleoside and GSH in Tris-HCl (■); nucleotide and GSH or mercaptoethanol in Tris-HCl (□); nucleoside and mercaptoethanol in Tris-HCl (▲).

philic attack on the 6-chloropurine nucleotide by an enzymic amino or hydroxyl group would have resulted in a 6-(alkylamino) or a 6-(alkoxy) purine derivative which have optical density maxima at 266-267 m and 248 m , respectively (79).

One may assume an average extinction coefficient for 6-(alkylmercapto) purine derivatives at 290 m of about 16,800 since the reported values for 6-methylmercaptopurine ribonucleoside and for 6-succinomercaptopurine ribonucleotide are 16,400 (78) and 17,200 (77), respectively. With this average extinction coefficient and the data in Figure 26, it is possible to calculate that the loss of 98% of enzyme activity was concomitant with a final concentration of 2.2×10^{-5} M 6-(alkylmercapto) purine nucleotide. The observed optical density at 290 m does not require correction for the absorption of 6-chloropurine nucleotide since at this wave length the extinction coefficient is only about 180. The twin peaked absorption spectrum of Figure 25-B should then resemble a mixture of 2.8×10^{-5} M 6-chloropurine nucleotide and 2.2×10^{-5} M 6-(alkylmercapto) purine nucleotide. At 263 m the molar extinction coefficient of 6-chloropurine nucleotide is 8400 (76) while a 6-(alkylmercapto) purine nucleotide would have a corresponding value of about 5200 (calculated from data in (77)). Hence, the calculated ratio of optical density at 290 m to that at 263 m would be 1.06 for this mixture of the two nucleotides. The observed ratio of 0.93 is in accord with the calculated value, and it is further evidence for the formation of a 6-(enzyme-mercapto) purine nucleotide derivative.

IMP dehydrogenase is inhibited by sulfydryl reagents (16, 19). This observation together with the fact that a thiol is a requirement for maximal activity demonstrates that a sulfydryl group(s) is required in

some manner for catalytic activity. IMP protects the IMP dehydrogenase of Sarcoma 180 tumor cells from alkylation by N-ethylmaleimide, iodoacetate, and iodoacetamide while NAD offers no such protection (19). This evidence also suggests that there is a sulfydryl group in the immediate area of the IMP binding site which can not be blocked without causing loss of enzymatic activity. As yet there is no evidence as to whether such a group is involved in binding or catalysis, or whether blocking this group prevents IMP having free access to its binding site. From the data in Figure 25, it may be calculated that 2.2 equivalents of 6-chloropurine nucleotide will almost completely inactivate 1 equivalent of IMP dehydrogenase of 200,000 molecular weight.

The rather unexpected results in Figure 27 show that the 6-chloropurine nucleoside is more readily attacked by thiols than is the nucleotide. One would, perhaps, not expect any significant differences in the reactivity of C-6 in these two derivatives of 6-chloropurine. This difference might be explained by a recent report on a NMR study of aqueous nucleotide solutions (52). In this report, the authors concluded that in aqueous solution nucleotides are predominantly in the anti conformation with the 5'-phosphate rotated up above the plane of the ribose ring and in close proximity to C-8 of the purine. This may have two effects on the rate at which a negatively charged sulfydryl attacks the electron deficient C-6 of 6-chloropurine nucleotide. First, an inductive effect at C-8 due to the negatively charged phosphate would decrease electron deficiency at C-6. Secondly, a negatively charged phosphate group in this position may retard the rate at which another negatively charged group attacks through simple electrostatic repulsive forces. Whatever the cause for the difference in reactivity, the results add additional evidence in

favor of the idea that the 6-chloropurine nucleotide inhibition of IMP dehydrogenase is site-specific as in a random bimolecular attack the nucleoside would be expected to be the more reactive species.

4. Inactivation Kinetics

a. Methods

It was found that at pH 8.1 the rate of IMP dehydrogenase inactivation by the 6-chloropurine nucleoside 5'-phosphate was too fast to measure by methods available in our laboratory. In order to slow the reaction to a convenient rate, the inactivation was studied at pH 7.0 in a 0.025 M potassium phosphate buffer.

Assay amounts of the highly purified IMP dehydrogenase were added to a solution (0.84 ml) that contained glutathione (2×10^{-3} M), KCl (0.1 M), and the phosphate buffer. After 5 minutes at 25°, the 6-chloropurine nucleotide was added to give the concentrations listed in the legend of Figure 28. After various intervals of time, the assay was started by addition of 0.16 ml of a mixture of IMP and NAD. Final concentrations of IMP and NAD were 4×10^{-4} M and 2×10^{-3} M, respectively. If these levels of substrates were added prior to the 6-chloropurine nucleotide, no enzyme inactivation was evident for at least 15 minutes. Hence, addition of the substrate mixture should stop enzyme inactivation and allow the extent of the inactivation to be determined at any given time.

The conversion of IMP to XMP was followed at 290 mμ and 25° with a Gilford spectrophotometer utilizing a recorder with full scale optical density deflections of 0.25 or 0.50. The uninhibited system had an optical density change of 0.35 in 5 minutes. Assays were linear during

the 5 to 10 minute assay period.

b. Results and Discussion

The inactivation of IMP dehydrogenase as a function of time followed first-order kinetics with a given concentration of 6-chloropurine nucleotide as evidenced by the log plots of Figure 28. The apparent first-order rate constants (k_{obs}) for the enzyme inactivations at different inhibitor concentrations may be obtained in reciprocal seconds when the slopes of the lines in Figure 28 are divided by sixty. These calculated pseudo first-order rate constants together with the corresponding inhibitor concentrations are: 8.24×10^{-3} sec^{-1}, 18.4×10^{-6} M; 9.84×10^{-3} sec^{-1}, 23×10^{-6} M; 12.6×10^{-3} sec^{-1}, 27.6×10^{-6} M; 15.4×10^{-3} sec^{-1}, 36.8×10^{-6} M; and 22.5×10^{-3} sec^{-1}, 61.3×10^{-6} M.

The progressive nature of the inhibitions suggested the formation of a covalent link between inhibitor and enzyme. There are two conceivable ways in which an inhibitor could react with the enzyme to form a covalent bond. The most direct way would be a random, bimolecular reaction between inhibitor and some enzymatic group. If this enzymatic group was involved in substrate binding, catalysis, or in maintaining protein structure, partial or complete inactivation may result. This type of covalent bond formation likely accounts for the effects of the general alkylating agents such as iodoacetate and the mercurials such as $HgCl_2$ or p-mercuribenzoate. The bimolecular reaction could be represented as in equation 8; where E is the catalytically active protein,

$$E + I\text{-}X \xrightarrow{k_1} E\text{-}I + X^- \qquad (8)$$

I-X is the inhibitor, E-I is the inactivated or partially inactivated enzyme and k_1 is the second-order rate constant describing this reaction.

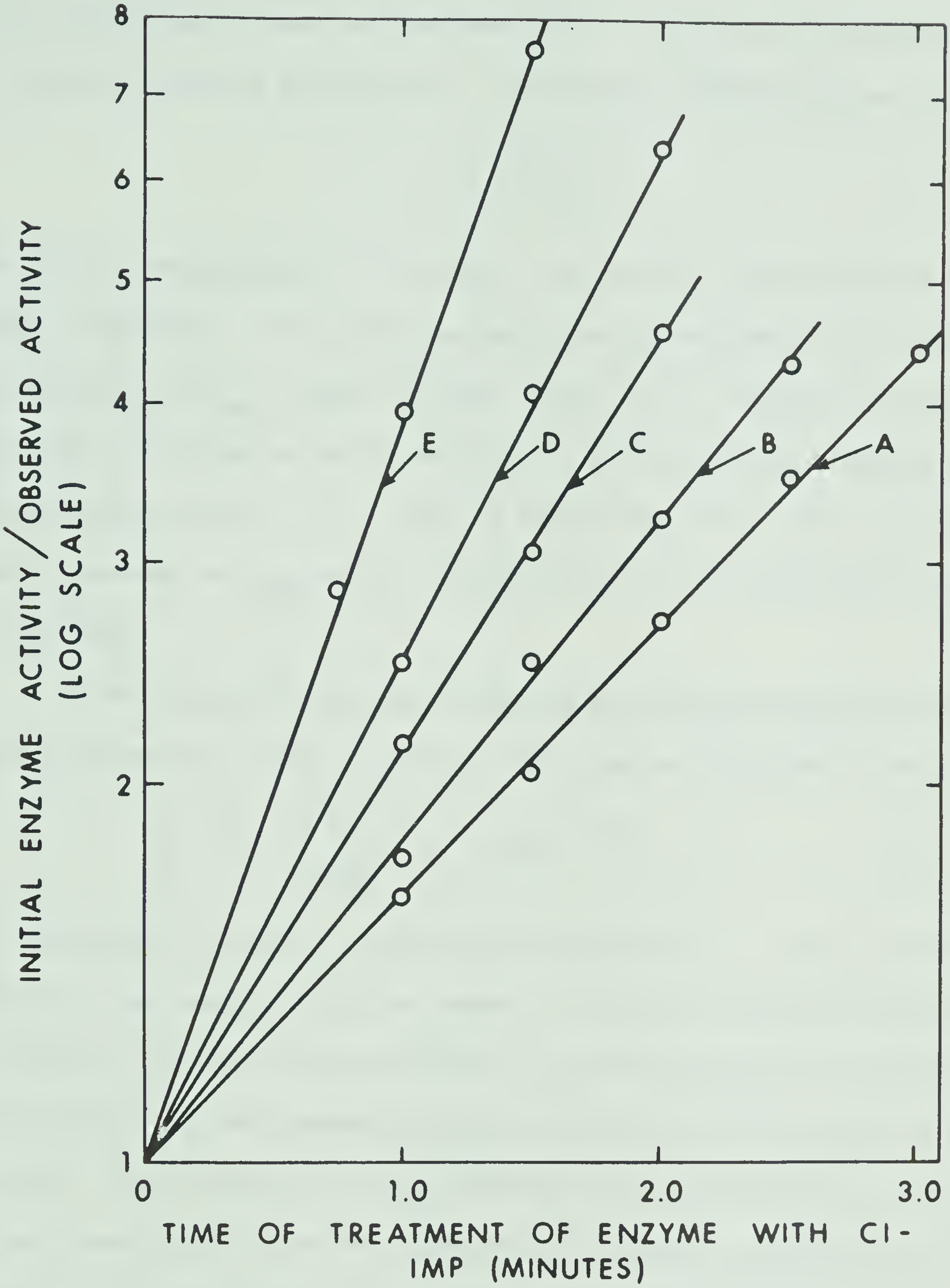


Figure 28: Rate of inactivation of IMP dehydrogenase by 6-chloropurine nucleotide at 25° and pH 7. Each point is the average of at least two determinations. Before addition of inhibitor the reaction velocity (change in absorbancy at 290 mμ) was 0.69 in 10 min. Concentrations of 6-chloropurine nucleotide; plot A, 18.4 μM; plot B, 23.0 μM; plot C, 27.6 μM; plot D, 36.8 μM; plot E, 61.3 μM.

This reaction may or may not be reversible. The rate of inactivation, R, would be given by equation 9. Since most inactivation experiments are

$$R = k_1 \left[E \right] \left[\text{I-X} \right] \qquad (9)$$

done with a vast excess of inhibitor, the rate of inactivation would be pseudo first-order. The experimentally determined pseudo first-order rate constants (k_{obs}) would be equal to $k_1 \left[\text{I X} \right]$. Hence, increasing the inhibitor concentration would result in a proportionate increase in the rate of inactivation. It is seen in Figure 29-A that there is not a linear increase in k_{obs} as the concentration of 6-chloropurine nucleotide is increased.

A covalent reaction between an inhibitor and an enzyme might also occur by a process shown in equation 10. Due to structural similarities

$$E + \text{I-X} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot \text{I-X} \xrightarrow{k_3} \text{E-I} + X^- \qquad (10)$$

with the normal substrate, an inhibitor may be able to form a reversible Michaelis-type complex with the enzyme. Additional reactive groups on the inhibitor which are not present on the substrate may be capable of reacting with various enzymatic groups that are in or very near the binding site. Such a reaction would prevent the inhibitor from dissociating and as a result give rise to a partial or, perhaps, complete inactivation. The rate of inactivation, R, for this model depends upon the concentration of the enzyme-inhibitor complex as seen in equation 11. The maximum rate

$$R = k_3 \left[E \cdot \text{I-X} \right] \qquad (11)$$

occurs when the concentration of this complex is as large as possible. This occurs when enough inhibitor is present to completely saturate the

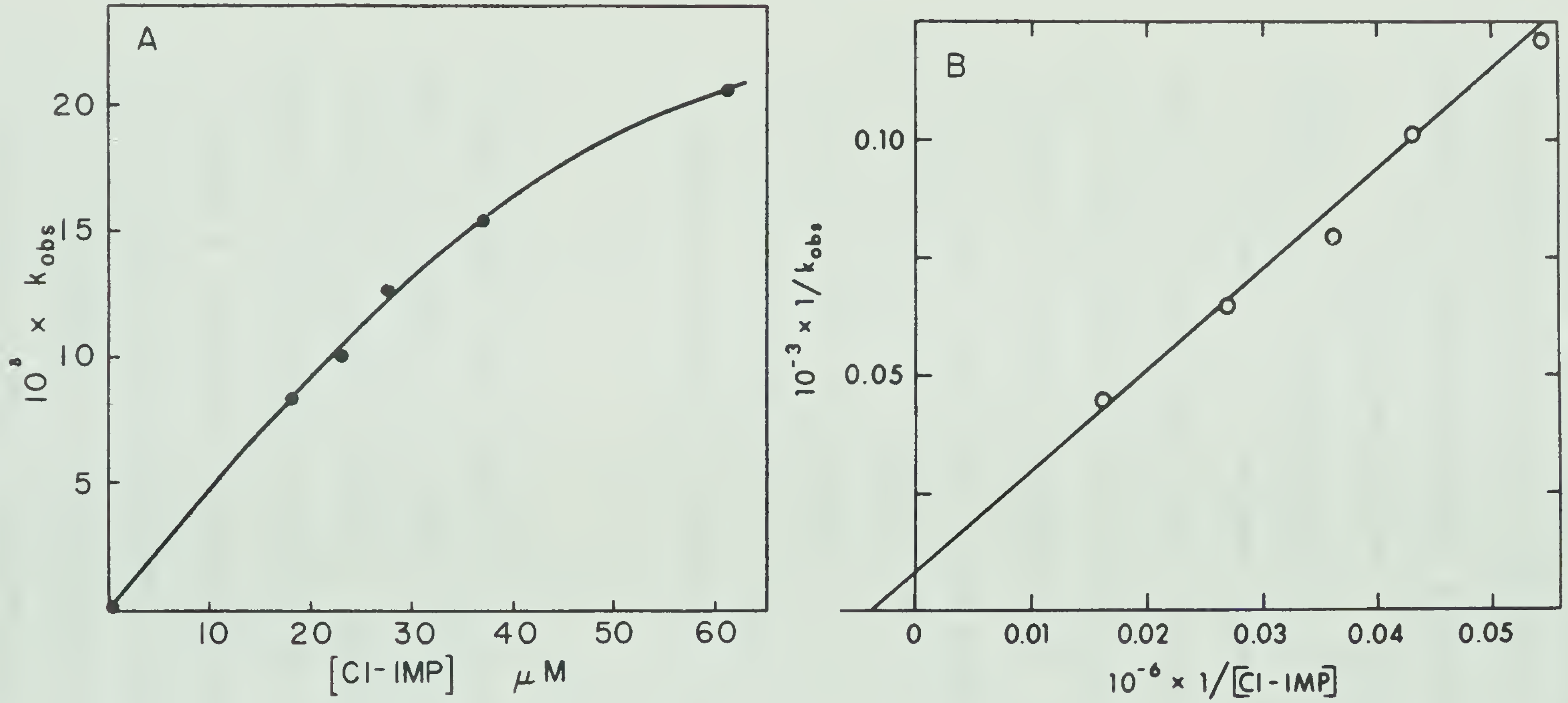


Figure 29: Frame A; rate constants for the IMP dehydrogenase inactivation plotted against the corresponding 6-chloropurine nucleotide concentrations. Frame B; data of Frame A plotted in reciprocal form. The k_{obs} values (sec^{-1}) were calculated from Figure 28.

enzyme. Any additional increase in the inhibitor concentration would then give no further increase in the inactivation rate. This limitation on the final inactivation rate has been referred to by Baker as a rate-saturation effect (80). In this case, the observed first-order inactivation rate constant (k_{obs}) would be expected to increase to a maximum value and then remain constant as the inhibitor concentration is raised.

Using equation 10 it is possible to derive equation 12 which relates the experimentally determined k_{obs} values to the inhibitor concentration and the various individual rate constants in this model (81, 82,

$$\frac{1}{k_{obs}} = \frac{K_{inact}}{k_3} \times \frac{1}{I} + \frac{1}{k_3} \qquad (12)$$

83). In this equation k_{obs} is the experimentally determined apparent first-order rate constant, I is the inhibitor concentration, k_3 is the actual first-order rate constant for the inactivation step of equation 10 and K_{inact} is equal to $(k_2 + k_3) / k_1$ (81, 83). Schaeffer *et al.* (82) replace K_{inact} with K_i which is simply the dissociation constant, k_2/k_1, for the inhibitor-enzyme complex. This difference results as Schaeffer does not take into account the concentration of inactivated enzyme (E-I, equation 10). Neglecting this species is only valid during the initial stages of inactivation when E-I is small compared to E. Equation 12 describes a straight line when $1/k_{obs}$ is plotted against $1/I$. From this type of plot the values for k_3 and K_{inact} may be determined.

If the data on the inactivation of IMP dehydrogenase by 6-chloropurine nucleotide is plotted in this manner, it is seen from Figure 29-B that a straight line does indeed result. This is strong evidence that the inactivation by this agent proceeds via an enzyme-inhibitor complex

[illegible]

[illegible]

$$[illegible]$$

[illegible]

[illegible]

[illegible]

as depicted by equation 10. This line was fitted by least squares, and values of 0.125 $\sec^{-1}$ and 2.6×10^{-4} M may be calculated from the slope and intercept of this line for the k_3 inactivation rate constant and for the K_{inact}, respectively. Application of this equation to other suspected intramolecular enzyme inactivations has led to values for k_3 of 0.052 $\sec^{-1}$ for phenylmethanesulfonyl fluoride and α-chymotrypsin (81), 0.0115 $\sec^{-1}$ for bromopyruvate and 2-keto-3-deoxy-6-phosphogluconic aldolase (83) and 0.0013 $\sec^{-1}$ for 9-(o-bromoacetamidobenzyl)adenine and adenosine deaminase (82). It is seen that the rates of these individual inactivations may vary over at least two orders of magnitude. In one case it is interesting to note that the K_{inact} value was in excellent agreement with the dissociation constant for this compound as determined by classical competitive inhibition studies (82). One may argue that the K_{inact} obtained for 6-chloropurine nucleotide is also a reasonable estimate of its dissociation constant with IMP dehydrogenase. Addition of the IMP and NAD to the preincubation mixtures of enzyme and 6-chloropurine nucleotide appeared to immediately stop further inactivation. The speed at which protection is established depends on the relative value of k_2 and k_3. If k_2 is small and much less than k_3, protection will not be set up as quickly since the inhibitor must first dissociate before IMP may bind and exert its protective influence. Assays were linear from the time they were started which was about 15 seconds after addition of the substrates. This indicates k_2 may be greater than k_3, and K_{inact} would reduce to k_2/k_1 which is the dissociation constant for the inhibitor. A value of 2.6×10^{-4} M for the dissociation constant of 6-chloropurine nucleotide and IMP dehydrogenase would be in the order of magnitude expected. Dissociation constants for IMP, XMP and GMP were not determined

at pH 7.0; but the IMP value would be in the range of 2.0 to 19.0 x 10^{-5} M, XMP in the range of 0.7 to 1.2 x 10^{-4} M, and GMP about 1.3 x 10^{-4} M as evidenced by the kinetic experiments at pH 8.1 and 6.0.

VII. INHIBITION AND INACTIVATION OF GMP REDUCTASE

1. Introduction

While the IMP dehydrogenase from *A. aerogenes* was being purified in our laboratory, it was observed that procedures used for the dehydrogenase also effected a purification of GMP reductase. It was seen in Chapter I that this enzyme has not been extensively studied. Since the reactions catalyzed by GMP reductase and IMP dehydrogenase are similar in that they both involve electron transfer at the C-2 position of a purine nucleotide, it seemed we had an opportunity to obtain some further information on the GMP reductase, and at the same time make a comparison of the effects of certain inhibitors and inactivators on both systems.

The first attempts at an initial velocity study on GMP reductase were not successful due to limitations in instrumentation. This problem together with the kinetic study which was eventually undertaken will be discussed in the next chapter. The first inhibition experiments on the GMP reductase showed that it like IMP dehydrogenase (20, 23) was progressively inactivated by the 6-chloropurine, 6-mercaptopurine, and 2-amino-6-mercaptopurine nucleoside 5'-phosphates. Additional experiments were undertaken to further compare these inactivations with respect to reversibility and substrate protection.

When the above experiments on crude cell extracts indicated that GMP reductase like IMP dehydrogenase may have an essential sulfydryl group in the region of the nucleotide binding site, more extensive studies utilizing these nucleotide inactivators and certain classical sulfydryl

reagents were carried out (84). Certain additional substrate analogues were screened to see if other potent inhibitors of this reaction could be found. It will be seen throughout the experiments described in this chapter that the information obtained subsequently from the kinetic data would have made interpretation of this data easier and in certain cases would have led to the design of different experiments.

2. Methods and Results

a. Sulfydryl Reagents

A step 4 GMP reductase preparation (Chapter II) which exhibited over 60% of total activity in the absence of glutathione was treated with p-chloromercuribenzoic acid (PMB) and the results are shown in Table VI. When PMB (Calbiochem) was added to the complete assay system less GSH, the resulting inhibitions occurred within 30 seconds which was as fast as could be measured. Addition of 2×10^{-3} M thiol reversed this effect with similar rapidity. After this rapid initial inactivation, there was no apparent additional inactivation for at least 30 minutes. If however the addition of the thiol to the PMB inhibited system was delayed for much more than 30 minutes, less and less of the activity became recoverable. Increasing the GMP concentration from 0.2×10^{-3} M to 5.0×10^{-3} M and/or increasing the TPNH concentration from 0.2×10^{-3} M to 0.6×10^{-3} M did not afford protection against the PMB. A 10 minute pretreatment of the enzyme with PMB in the absence of GMP and NADPH gave similar inhibitions to those of Table VI.

Iodoacetamide and iodoacetate (Calbiochem) inhibited GMP reductase progressively during preincubations (Figure 30). When 2×10^{-3} M GMP was present, the inactivation did not exceed 11% during the time

TABLE VI: Inhibition of GMP Reductase by PMB.[a]

PMB Concn (μM)	GSH Concn (mM)	Rel Act.
0	2.0[b]	100
0	0	62
1	0	47
2.5	0	16
10	0	0
10	2.0[c]	91

[a] The PMB was added to the complete assay mixture (GSH omitted) 5 min after the reaction was started.
[b] GSH included in assay mixture prior to addition of PMB. [c] GSH added to PMB-inhibited system 5 min after PMB addition.

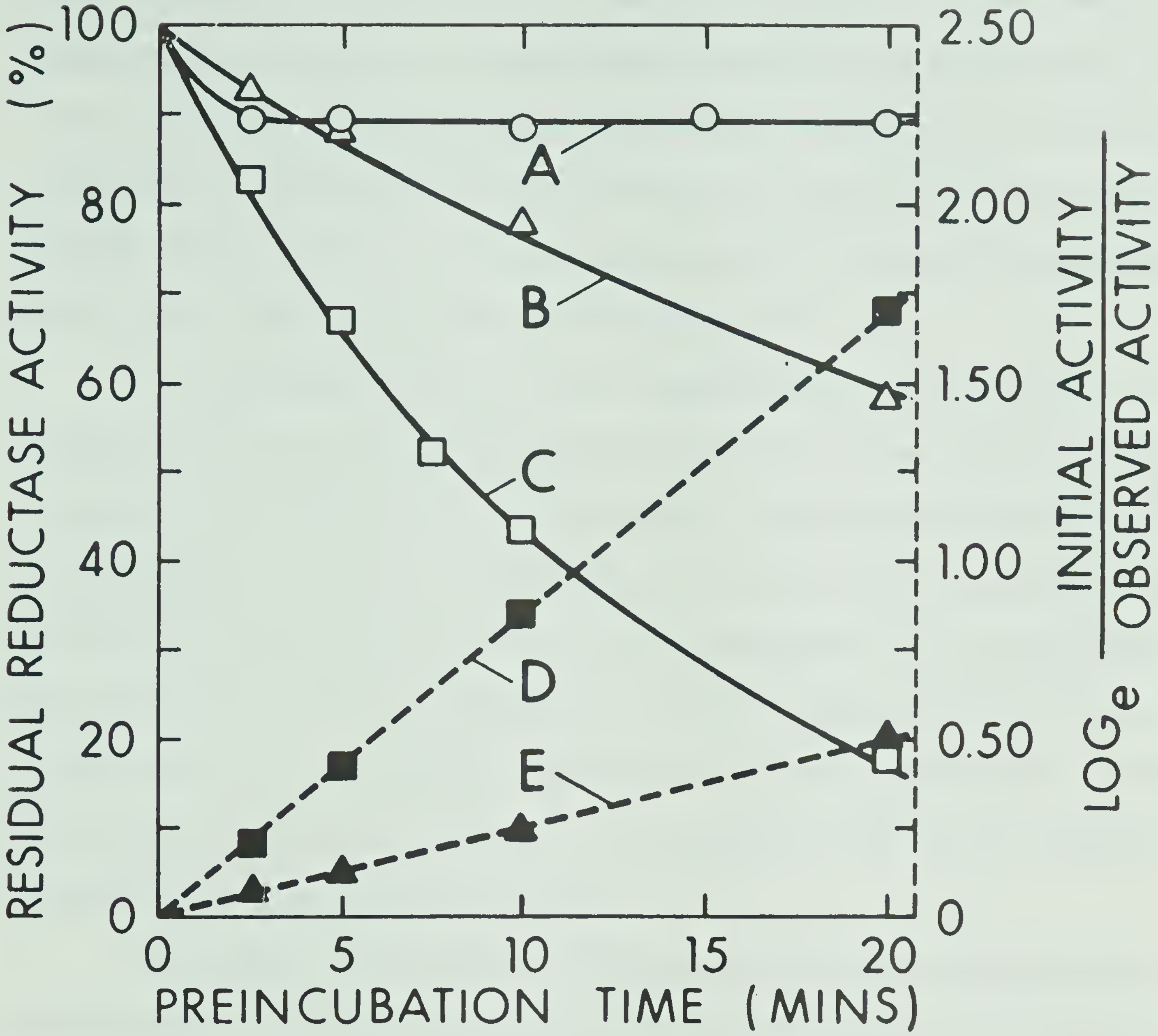


Figure 30: Inactivation of GMP reductase by 500 μM iodoacetamide or iodoacetate. Step 4 enzyme (25 μl) was preincubated at 23° with the iodo compounds in assay mixtures which lacked GMP and/or NADPH, as indicated. Curve A: effect of iodoacetamide or iodoacetate in the presence of 2 mM GMP (mixtures assayed after addition of NADPH). Curve B: effect of iodoacetate in the absence of GMP. Curve C: effect iodoacetamide in the absence of GMP. Curve D: logarithm plot of the data of curve C. Curve E: logarithm plot for curve B. In the absence of inhibitors the decrease in optical density at 340 mμ was no less than 0.06 in 10 min.

shown. NADPH at 6×10^{-4} M did not protect the enzyme. These iodo compounds also reacted with the glutathione present in the assay mixture, and it was estimated from published data (85) that the half lives at the concentrations involved would be 25-35 minutes for iodoacetamide and 65-75 minutes for iodoacetate. The rate of inactivation appeared to be described by first order reaction kinetics (Figure 30).

The inactivation was not due to depletion of glutathione as experiments with 1.5×10^{-3} M glutathione gave less than a 10% reduction in rate. This concentration of glutathione would represent the level of thiol remaining if all the iodoacetate had reacted with glutathione. If 5×10^{-4} M iodoacetamide and 2.0×10^{-3} M glutathione were preincubated for 15 minutes before an experiment as shown in Figure 30 was carried out, it was seen that a 15 to 20% decrease in the rate of inactivation occurred. This shows that the iodoacetamide is the inhibitor and not the iodoacetamide-alkylated glutathione compound.

The reaction was pseudo first-order and the rate constant obtained from first-order log plots includes the concentration of alkylating agent. The calculated half life for iodoacetamide and glutathione at the concentrations in question was only slightly larger than the longest preincubation time. Hence, there had been perhaps a 25% reduction in iodoacetamide concentration after the 20 minute preincubation experiment. The actual inactivating ability of iodoacetamide relative to iodoacetate is then slightly better than that depicted in Figure 30.

b. Progressive Inactivation by GMP Analogues

Preliminary experiments were carried out with the crude Fraction A extract of *A. aerogenes* (Chapter II). This fraction did not require additional glutathione but final assay mixtures contained 1.3×10^{-4} M

β-mercaptoethanol due to the presence of this thiol in the enzyme preparation. Preincubation mixtures contained inhibitor and assay components minus the GMP. The GMP was added after a specified time to start the reaction. The synthesis of the 2-amino-6-mercaptopurine and the 6-mercaptopurine nucleoside 5'-phosphates has been previously described (76).

The 6-chloropurine nucleotide at 9.2×10^{-5} M gave 47 and 86% inactivation in preincubations of 5 and 10 minutes, respectively. At a concentration 9×10^{-6} M, 2-amino-6-mercaptopurine nucleotide gave inactivations of 55 and 80% in the 15 and 30 minute preincubations. In an experiment in which the GMP was added simultaneously with 1.0×10^{-4} M 6-mercaptopurine nucleotide there was observed a time-dependent inactivation with a 25% loss of activity within 15 minutes. GMP at 1×10^{-3} M offered protection against these above inactivations if added prior to the inhibitor but had no effect once inhibition had occurred.

The progressive nature of the inhibition when 6-chloropurine nucleotide is added to the complete assay mixture containing the more purified step 4 GMP reductase is seen in Figure 31. Preincubation of the enzyme with the 1×10^{-5} M level of 6-chloropurine nucleotide also results in a time-dependent inactivation of GMP reductase (Figure 32-A). Including GMP at a level of 1×10^{-3} M in the preincubation mixtures effectively protected the enzyme from inactivation (Figure 32-D), however, the inactivation once established was not reversed by 1×10^{-3} M GMP. GMP at a concentration of 2×10^{-4} M was less effective as a protective agent (Figure 32-B). Whereas neither NADPH at 6×10^{-4} M nor GMP at 5×10^{-5} M alone afforded protection, the combination of these reagents did slow the inactivation (Figure 32-C).

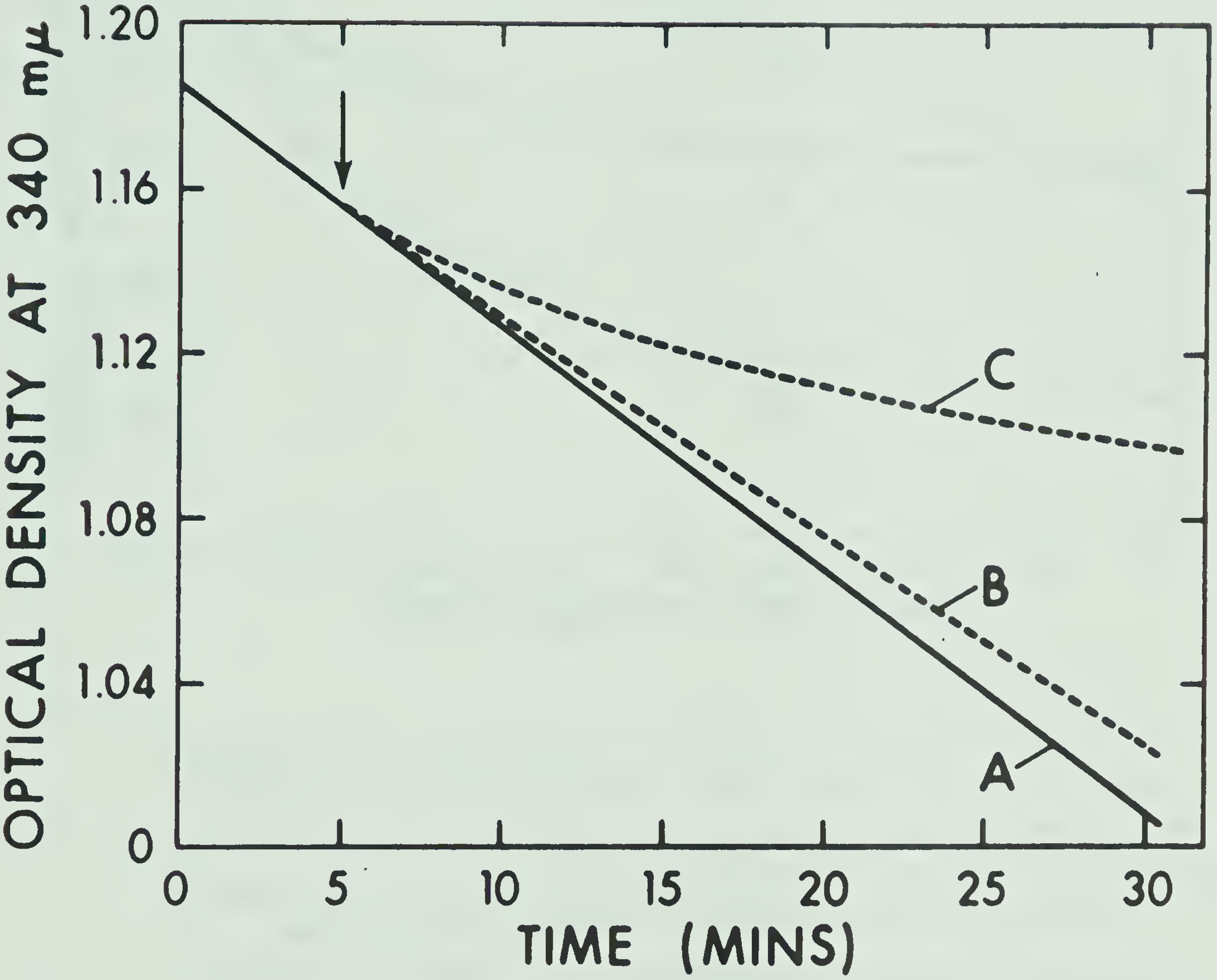


Figure 31: Inactivation of GMP reductase by 6-chloropurine nucleotide. Step 4 enzyme (25 μl) was employed and nucleotide was added (arrow) to the standard assay mixture. Curve A: no inhibitor added. Curve B: 10 μM 6-chloropurine nucleotide. Curve C: 190 μM 6-chloropurine nucleotide.

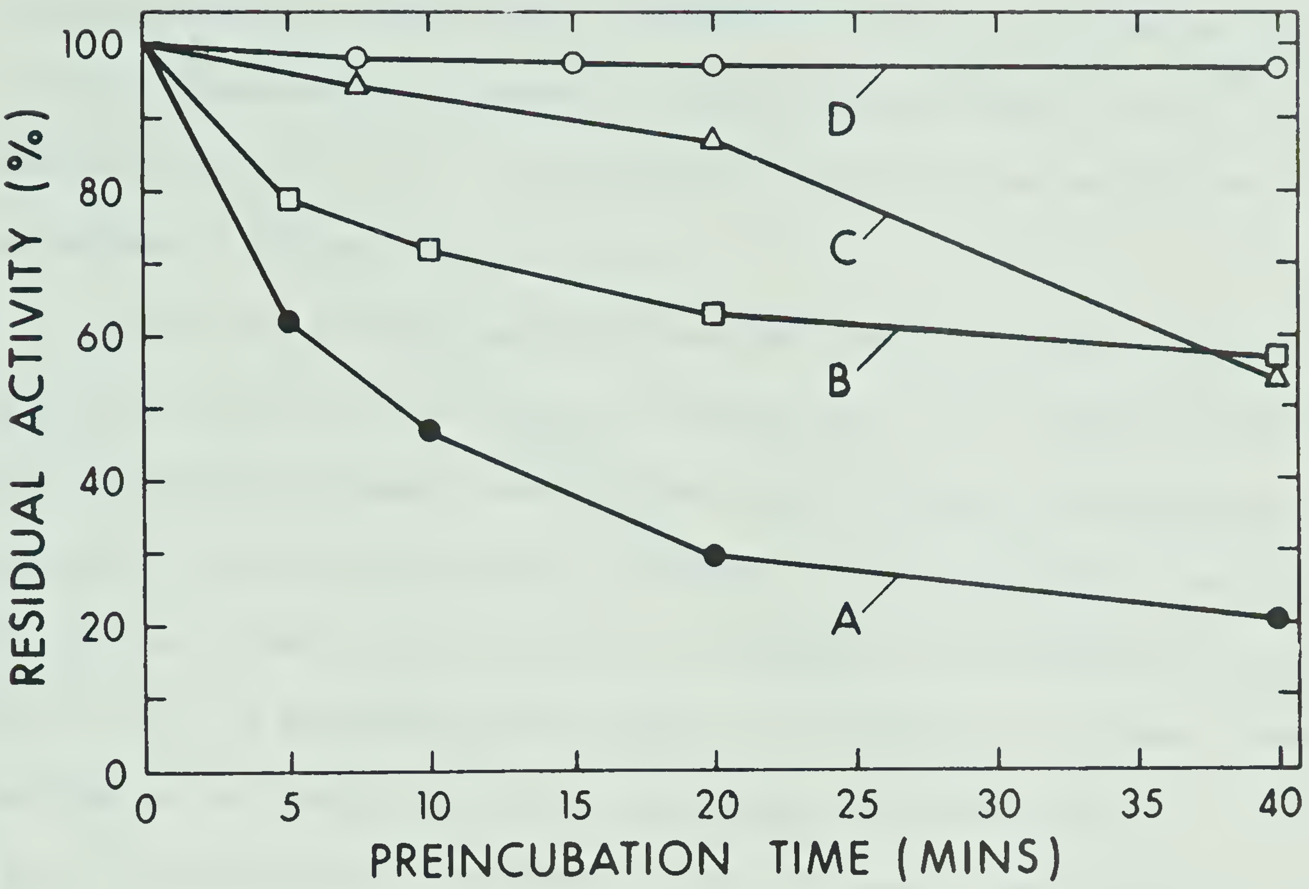


Figure 32: Inactivation of GMP reductase by 10 μM 6-chloropurine nucleotide. The enzyme (25 μl of step 4 fraction) was exposed at 23° to the nucleotide in the assay system. Plot A, preincubation conditions as follows: (a) GMP and NADPH omitted, (b) GMP omitted and 600 μM NADPH included, or (c) NADPH omitted and 50 μM GMP included. Plot B, NADPH omitted and 200 μM GMP included. Plot C, 600 μM NADPH and 50 μM GMP included and enzyme activity measured after addition of more GMP (final concentration, 200 μM). Plot D, NADPH omitted and 1 mM GMP included.

A step 4 enzyme preparation which had no activity in the absence of glutathione was treated for 20 minutes in the assay buffer with 3.4×10^{-4} M 6-chloropurine nucleotide. Upon addition of GMP (1×10^{-3} M), glutathione (2×10^{-3} M), and NADPH (2.0×10^{-4} M), no inhibition over the untreated control was observed.

With step 4 enzyme preparations that retained much of their enzymatic activity in the absence of added thiol, the 2-amino-6-mercaptopurine nucleotide brought about at low concentrations a progressive inhibition of the GMP reductase. The rate of this progressive inactivation was reduced by GMP (Figure 33) but was unaffected by NADPH at 6×10^{-4} M. When 2×10^{-3} M glutathione was added to the inhibited systems, greater than 90% of the maximal activity was regained within 5 minutes. If 2×10^{-3} M glutathione was present under the conditions of Figure 33, a nonprogressive inhibition of 5-10% occurred. A similar type of progressive inhibition which could be retarded by GMP and reversed by 2×10^{-3} M glutathione was observed with 6-mercaptopurine nucleotide (Figure 34).

The 6-chloropurine and 2-amino-6-chloropurine nucleosides at concentrations of 4×10^{-3} M gave time-independent inhibitions of 22 and 10% respectively.

c. Inhibition by Other Nucleotides

The step 4 reductase was strongly inhibited by ATP. With a two-fold excess of ATP over GMP (4×10^{-4} M vs. 2×10^{-4} M) an inhibition of 90 to 95% was observed. This could seemingly be completely reversed by increasing the GMP to 4×10^{-3} M. An inhibition of some 85% was also obtained with ADP, but a ten-fold excess of ADP (2×10^{-3} M) was required. The inhibition could be completely reversed by 3×10^{-3} M GMP. A ten-fold excess of GTP (2×10^{-3} M) gave only 20% inhibition. The ADP, ATP

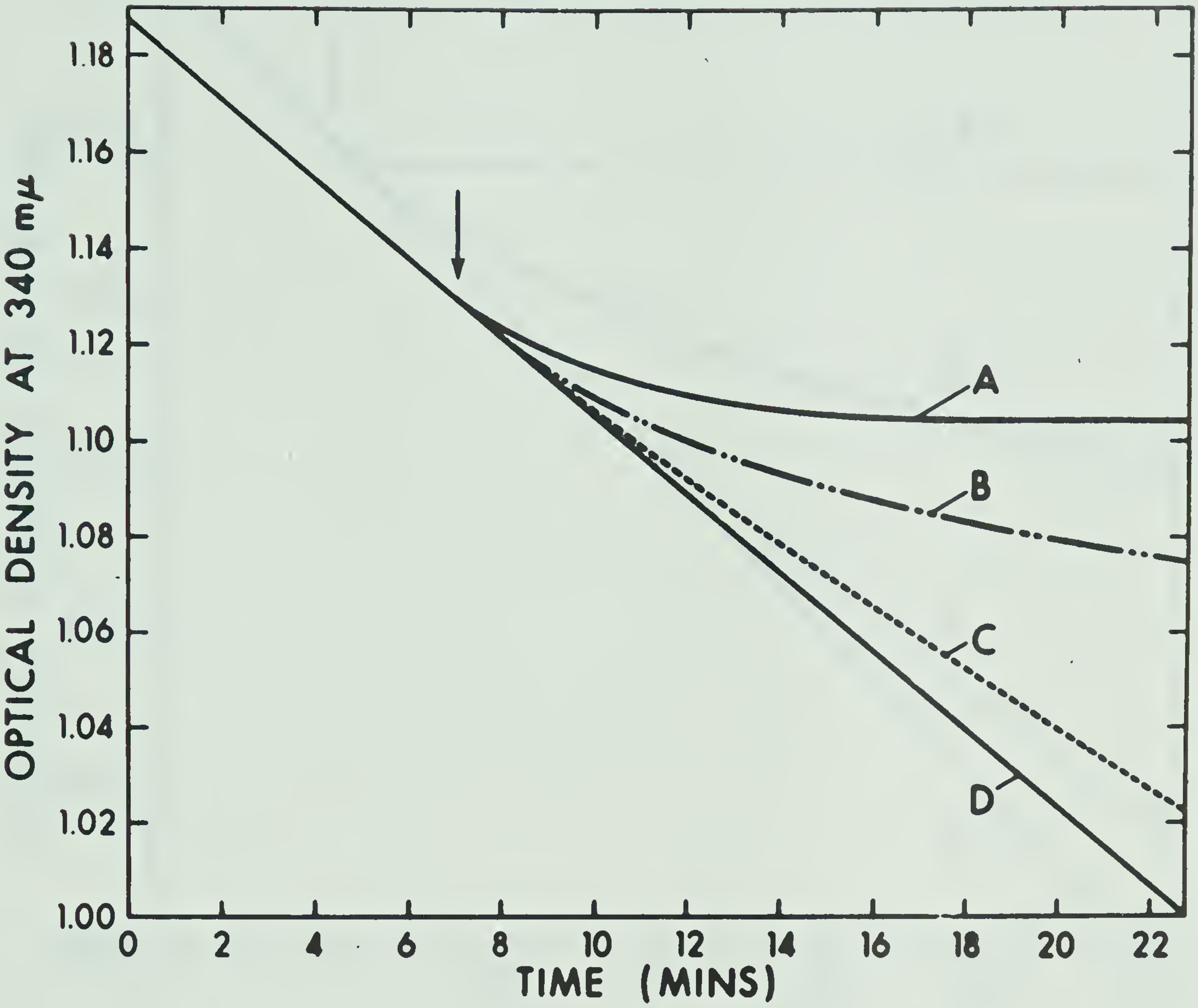
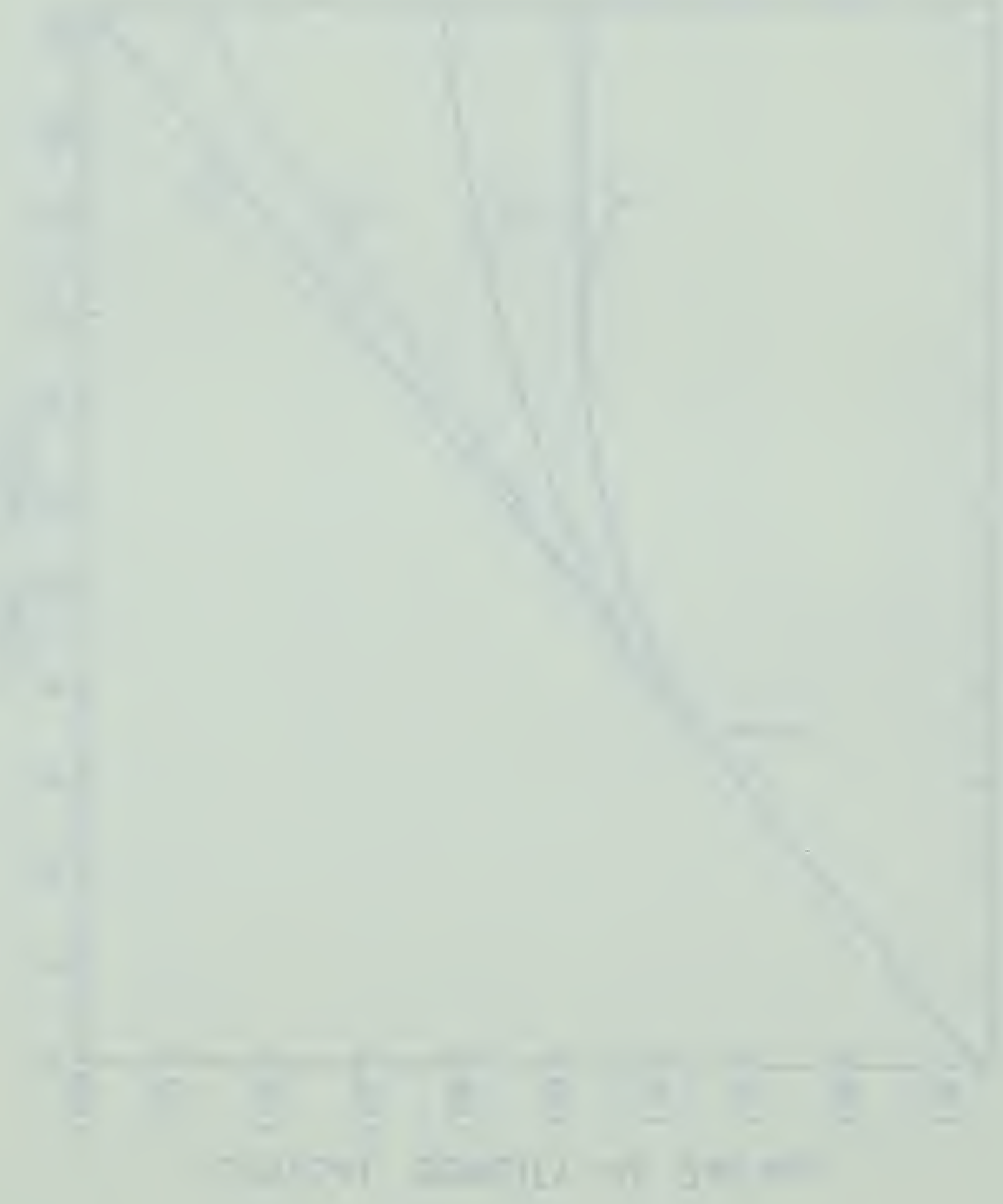


Figure 33: Inactivation of GMP reductase by 15 μM 2-amino-6-mercaptopurine nucleotide. The step 4 enzyme preparation employed (50 μl/assay) was not stimulated by GSH. The inhibitor was added (arrow) to assay mixtures which lacked GSH and contained the following initial concentrations of GMP: curve A, 100 μM; curve B, 500 μM; and curve C, 2 mM. Curve D was obtained under conditions A-C in the absence of inhibitor.



[illegible]

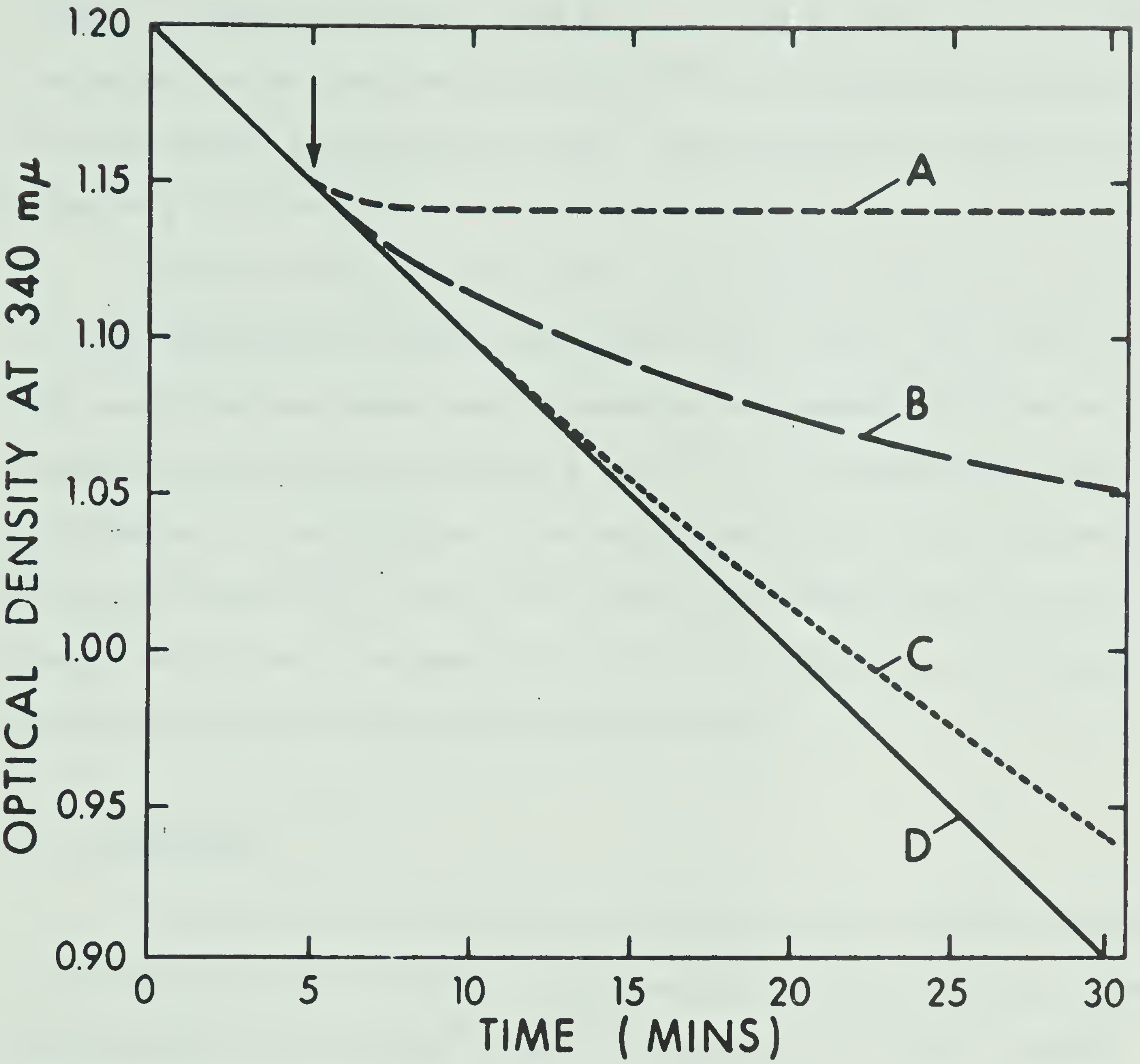


Figure 34: Inactivation of GMP reductase by 2.5 μM 6-mercaptopurine nucleotide. Conditions were as in Figure 33. Initial concentrations of GMP were: curve A, 50 μM; curve B, 250 μM; and curve C, 2 mM. Curve D was obtained in the absence of inhibitor.

and GTP were added as sodium salts and no divalent cation was included. Table VII shows the effect of ATP at 4 x 10^{-4} M and GTP at 1 x 10^{-3} M on the extent of the inactivation by 5 x 10^{-5} M 6-chloropurine nucleotide in a ten minute preincubation at 24°. The reaction was started by addition of 5 x 10^{-3} M GMP.

Various additional nucleotides were tested to see if any inhibited the GMP reductase as strongly as did ATP (Table VIII). The inhibitors at the concentrations shown were included in the standard reductase assay containing both GMP and NADPH at 2 x 10^{-4} M. All assays were linear with no progressive inactivations. The nucleotides used in this section were obtained commercially from either Sigma or Calbiochem except for the purine nucleoside 5'-phosphate (93) and the 2'-IMP which was obtained from the oxidative deamination of 2'-AMP (94).

3. Discussion

The results with PMB and the iodoacetates show that GMP reductase can also be placed in that very large and very general group of enzymes that require free sulfydryl groups for enzymatic activity. The PMB results are somewhat unique in that the inhibition is established very rapidly and seems to be reversed as quickly (86). It was not possible due to the speed of the reaction to study more definitively the rate of inactivation with methods available to us in our laboratory. From Webb's review (86) it appears that in the majority of cases the blocking of the most reactive enzyme sulfydryl groups by mercurials does not lead to loss of activity. Only after the more sluggish sulfydryls start reacting is activity lost. For instance, eight to ten of aldolase's sulfydryls have reacted before activity starts to disappear (88). However, there are

TABLE VII: Effect of ATP and GTP on the Inactivation by 6-Chloropurine Nucleotide.[a]

Triphosphate	% Inhibition
0	80
0[b]	9
ATP	82
GTP	79
ATP[c]	0

[a] See text for reaction conditions.

[b] GMP included in preincubation.

[c] 6-Chloropurine nucleotide omitted.

TABLE VIII: Inhibition of GMP Reductase.

Inhibitor	Concentration (mM)	% Inhibition
Purine 5'-nucleotide	0.6	0
2'-IMP	2.0	0
5'-IMP	2.0	40
5'-AMP	1.0	20
2'-AMP	1.0	48
2'-(3')-GMP	2.5	0

definitely cases such as succinate dehydrogenase (89) in which activity disappears along with the reaction of the initial sulfydryl groups. GMP reductase likely belongs in the latter group.

The observation that less and less activity became recoverable as the time of PMB treatment increased may have two explanations. Additional sulfydryl groups may be starting to react, and it is the blocking of these groups which gives rise to the irreversible effects. The alternative explanation is that there is a time-dependent irreversible effect due to the introduction into the protein of the rather large and negatively charged benzoic acid moieties. A similar situation has been described for phosphoglyceraldehyde dehydrogenase in which the extent of reversibility by cysteine was dependent upon the length of exposure to the mercurial (90). The irreversibility in this latter case was attributed to a time-dependent unfolding of the polypeptide due to the introduction of the mercurial. This was evidenced by a corresponding change in the ORD pattern and viscosity (90).

It is generally assumed that the iodoacetates are specific for sulfydryl groups when concentrations of less than 1×10^{-3} M are employed at room temperature and neutral pH (87). These reagents will, however, also react with amino groups (91) and the sulfur group of methionine (92). The latter reaction would be the most likely at physiological temperature and pH. The rapid disappearance of activity in the presence of PMB adds support to the contention that loss of activity with the iodoacetates is also the result of reaction with sulfydryl groups. The observation that iodoacetamide is a more effective inactivating agent of GMP reductase than is iodoacetate can not be attributed to the charge difference since the negatively charged PMB inactivates, though, conceivably not at the

same site. Webb has stated that iodoacetate and iodoacetamide inhibitions develop slowly and seldom reach completion before 30-60 minutes (87). If this is so, the acetate inhibitions of GMP reductase are relatively fast as was the PMB induced effect. This is consistent with these reagents reacting at the same critical point or points.

One would perhaps not expect the negative charge of PMB or iodoacetate to influence the approach of these reagents to the GMP binding site since GMP itself is negatively charged. The difference in the reactivity of these acetate derivatives has been observed by many workers. For instance Smythe (85) reported that at pH 7.1 iodoacetamide was 1.38 and 1.83 times as effective as iodoacetate in alkylating cysteine and glutathione.

From the PMB and acetate derivative results one might draw the rather general conclusion that there is a free sulfydryl group either in or vicinal to the GMP binding site of the reductase. Blockage of this group causes loss of activity but it can not be determined from present evidence whether this occurs because the group is functional or is simply due to steric hindrance at the GMP site.

The patterns of progressive inactivations of GMP reductase by the 6-chloropurine, 2-amino-6-mercaptopurine and 6-mercaptopurine nucleotides are of the same type as was observed with the IMP dehydrogenase from the same bacteria (20, 23). The inactivations could be retarded by GMP but once the inhibition was established GMP could not effect a reversal. As was the case with the IMP dehydrogenase, the pyridine coenzyme, in this instance NADPH, did not appear to affect the rates of inactivation with these synthetic nucleotides.

The corresponding nucleosides were much less effective as inhib-

itors and paralleling this it has been observed that guanosine has little inhibitory effect (21). It seems that the phosphate group is required for both GMP binding and also for inactivation by these chloro and mercaptopurine derivatives. Apparently binding of these inhibitors is a prerequisite for inactivation. One would expect these synthetic compounds to have some affinity for the GMP binding site since they are close structural analogues of either GMP or of the product IMP. The observation that inactivation by chloropurine nucleotide does not occur in the absence of thiol with enzyme preparations exhibiting an absolute thiol requirement again supports the idea that this agent is GMP site-specific.

A progressive inhibition suggests some type of covalent bond formation. The most reactive positions of the inhibitory nucleotides are the electron deficient C-6 of 6-chloropurine nucleotide or the 6-mercapto moiety of the 2-amino-6-mercaptopurine and the 6-mercaptopurine nucleotides with its easily donated electron pair. The inhibition most likely proceeds, as is believed to be the case with IMP dehydrogenase, by means of a nucleophillic attack of an enzymatic sulfydryl group on the C-6 of the chloro derivative, and through disulfide bond formation with the 6-mercapto analogues. The case for the mercapto analogues is supported by the preventive or reversal properties of glutathione, depending upon whether it is added before or after inactivation has occurred. The results with PMB and the iodoacetates are consistent with the above postulations in that they indicate a sulfydryl group may indeed be located in the immediate vicinity of the GMP binding site.

On protein evolutionary grounds, it might be expected that the binding sites of various enzymes for nucleoside monophosphates may have some common denominators. The results with both the IMP dehydrogenase

and GMP reductase suggest a free sulfydryl group is either in or very close to the nucleotide binding site. As the position of this group is very critical if one postulates it to be able to attack the C-6 or C-6 sulfur of the inhibitory nucleotides, it becomes less likely that such a group occurs in this position in two enzymes without a specific function. Whether it is of structural or of catalytic importance is unresolved. Whatever the possible function of such a sulfydryl group, its reactivity has not been greatly altered by the partial purification since results with these inactivating nucleotides are qualitatively similar for the crude preparations and the phosphocellulose purified enzyme.

As reported in the earlier study on the bacterial GMP reductase (21), ATP was also a strong inhibitor of our step 4 reductase preparations, whereas GTP was much less inhibitory. This inhibition seemed completely competitive with GMP, while NADPH could not be demonstrated to compete with ATP. Magasanik postulated that this ATP inhibition could be a control feature since if sufficient cellular levels of ATP existed there would be no reason to channel guanine nucleotides to adenine nucleotides (21).

The site at which ATP is bound would not be expected to be the GMP site since one is a triphosphate and the other a monophosphate, and GTP while being a closer analogue of GMP is a much weaker inhibitor. The fact that GMP and ATP seemed mutually competitive could be explained if both bound to the same enzyme form but at different sites. The structural similarity between ATP and NADPH would suggest binding might occur at the pyridine coenzyme site, but this tends to be ruled out by the lack of a demonstrable NADPH effect on the ATP inhibition. The in-

ability of ATP to protect the GMP reductase from the 6-chloropurine nucleotide, as did GMP, implies strongly that these two nucleotides react at different sites. One is left with the alternative that a specific regulatory site could exist for ATP.

The method of expressing the inhibitions shown in Table VIII is admittedly not the best way of comparing inhibitors. In such a case one is comparing velocities at arbitrary substrate concentrations without any concern for whether competitive, noncompetitive, or uncompetitive inhibition is involved, and thus, one has no real idea as to whether the observed differences are reflecting actual binding or more complex situations. However, as mentioned earlier, kinetic experiments were not possible at the time of these studies, but it was of interest to ascertain in at least a general way as to which, if any, mononucleotides might be as inhibitory as ATP.

None of those tested were as effective at inhibiting the GMP reductase reaction as was ATP. The 5'-IMP was an inhibitor as expected, since it is one of the products. This inhibition will be further discussed in the next chapter. The lack of inhibition by either 2'-IMP or the mixed 2' and 3'-phosphates of GMP demonstrate that not only the presence of a phosphate group but also its position is important in the binding of the monophosphates to GMP reductase. The effects of the adenine mononucleotides are difficult to discuss with data of this type. They may have affinity for the GMP or NADPH site and possibly the postulated ATP site. It would seem though that some binding at the NADPH site occurs since 2'-AMP is more effective than 5'-AMP at the same concentration. The lesser inhibitions effected by the mononucleotides as compared to ATP and ADP tend to further support the idea of a separate site for these compounds.

VIII. KINETICS OF GMP REDUCTASE

1. Introduction

There has not been any comprehensive kinetic study reported on the GMP reductase from any source. For this reason alone, it became of interest to examine this system utilizing contemporary kinetic theory. As discussed earlier, such kinetic data usually permits the selection of a working kinetic model (Chapter IV). The results of the last chapter should then be consistent with any such model. For instance, it was postulated that certain of the synthetic nucleotides inactivated GMP reductase by first binding to the GMP site and then reacting covalently with the enzyme. Since this inactivation occurred in the absence of NADPH, the above postulate requires that GMP must itself bind to the reductase in the absence of NADPH. The kinetic model would be expected to substantiate this. The kinetic analysis might also suggest why inactivation by 6-chloropurine nucleotide proceeded slower under reaction conditions (Figure 32).

Prior to carrying out much of the work described in the last chapter, kinetic experiments had been attempted. It had become apparent at this time that the Michaelis constants for GMP and NADPH were both very low and probably of the order of 1.5×10^{-5} M. The lack of sensitivity in the recorder being used with a Gilford spectrophotometer prevented reliable measurements of the small optical density changes involved. The kinetic study was subsequently undertaken when it was found that reproducible results might be obtained using a Cary model 15 spectrophotometer with a full scale recorder deflection of 0.10 optical density unit.

2. Results and Discussion

A glutathione-dependent GMP reductase preparation isolated by phosphocellulose column chromatography as described in Chapter II was used. This sample contained six main protein bands with perhaps three additional trace bands when examined with polyacrylamide gel electrophoresis. Which band or bands contained the reductase is not known. There was no GMP-independent NADPH oxidase activity in this preparation. The GMP and NADPH were not broken down by these extraneous proteins as evidenced by the lack of optical density changes when either substrate was preincubated in the assay mixture for 30 minutes. Furthermore, this preincubation did not reduce the initial velocity.

Figures 35 and 36 show the reciprocal plots of the initial velocity data at pH 7.5. An intersecting pattern is obtained with both the slopes and intercepts being linear functions of the reciprocals of the fixed substrate concentrations. From Figures 37 and 38 it is seen that IMP is a linear competitive inhibitor of GMP, and NADP is also a linear competitive inhibitor of NADPH.

The linear intersecting pattern of the reciprocal plots is consistent with either an ordered or a random rapid-equilibrium type of kinetic model (40). An ordered model would predict that the last product to be released should be competitive with the first substrate added, whereas other combinations of products and substrates should give noncompetitive or uncompetitive inhibition (53). The random rapid-equilibrium models predict that all combinations of products and substrates will give linear competitive inhibition when it is assumed there is no formation of deadend complexes (54). The product inhibition data although not complete is consistent with the random kinetic model. No attempt

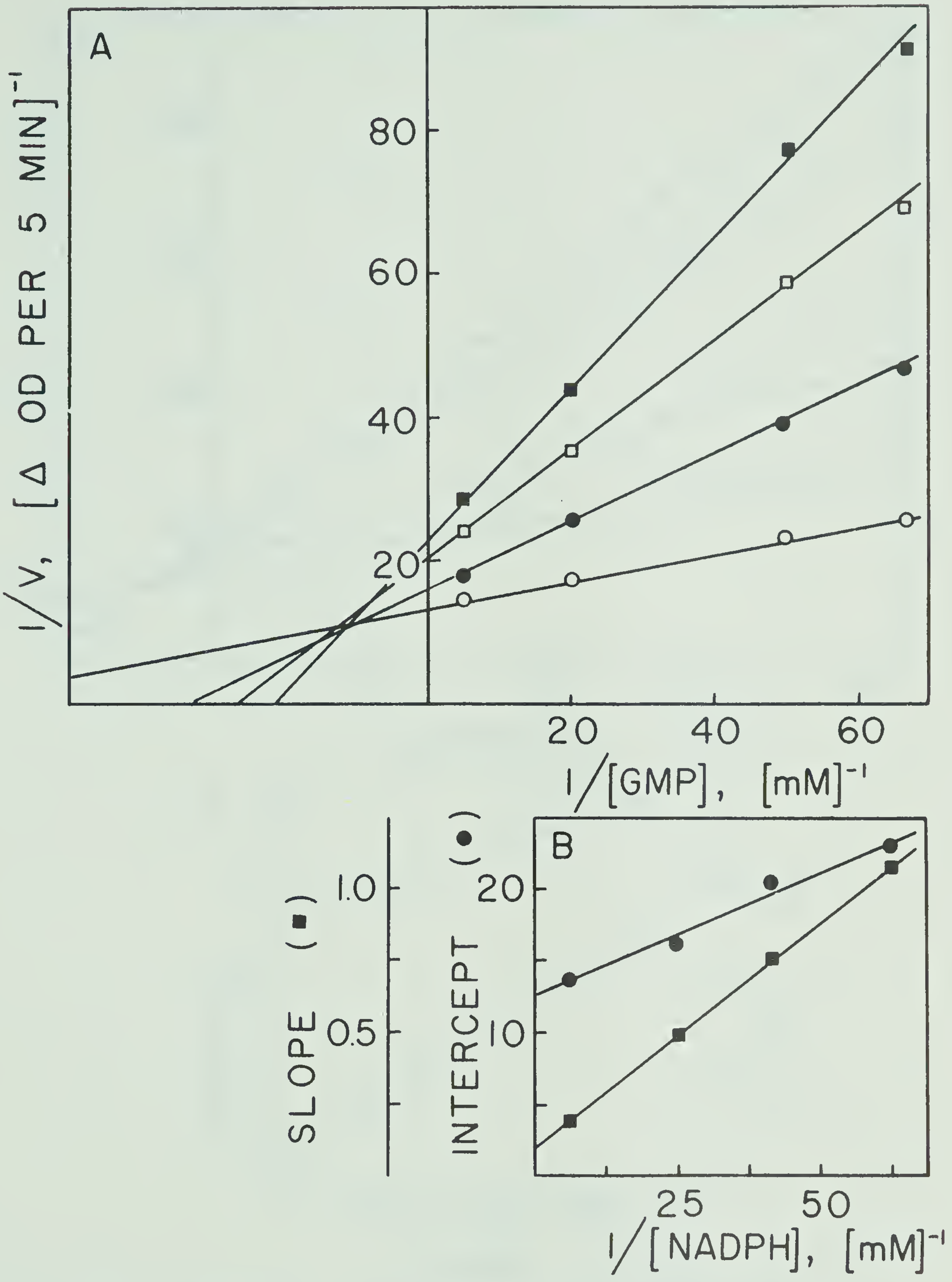


Figure 35: Initial velocity data for GMP reductase. Frame A; GMP varied at fixed NADPH concentrations of 1.6 x 10^{-5}M (■), 2.4 x 10^{-5} M (□), 4.0 x 10^{-5} M (●), and 1.6 x 10^{-4} M (○). Frame B; slopes and intercepts replotted against the reciprocal of the NADPH concentrations. OD changes measured at 340 mμ.

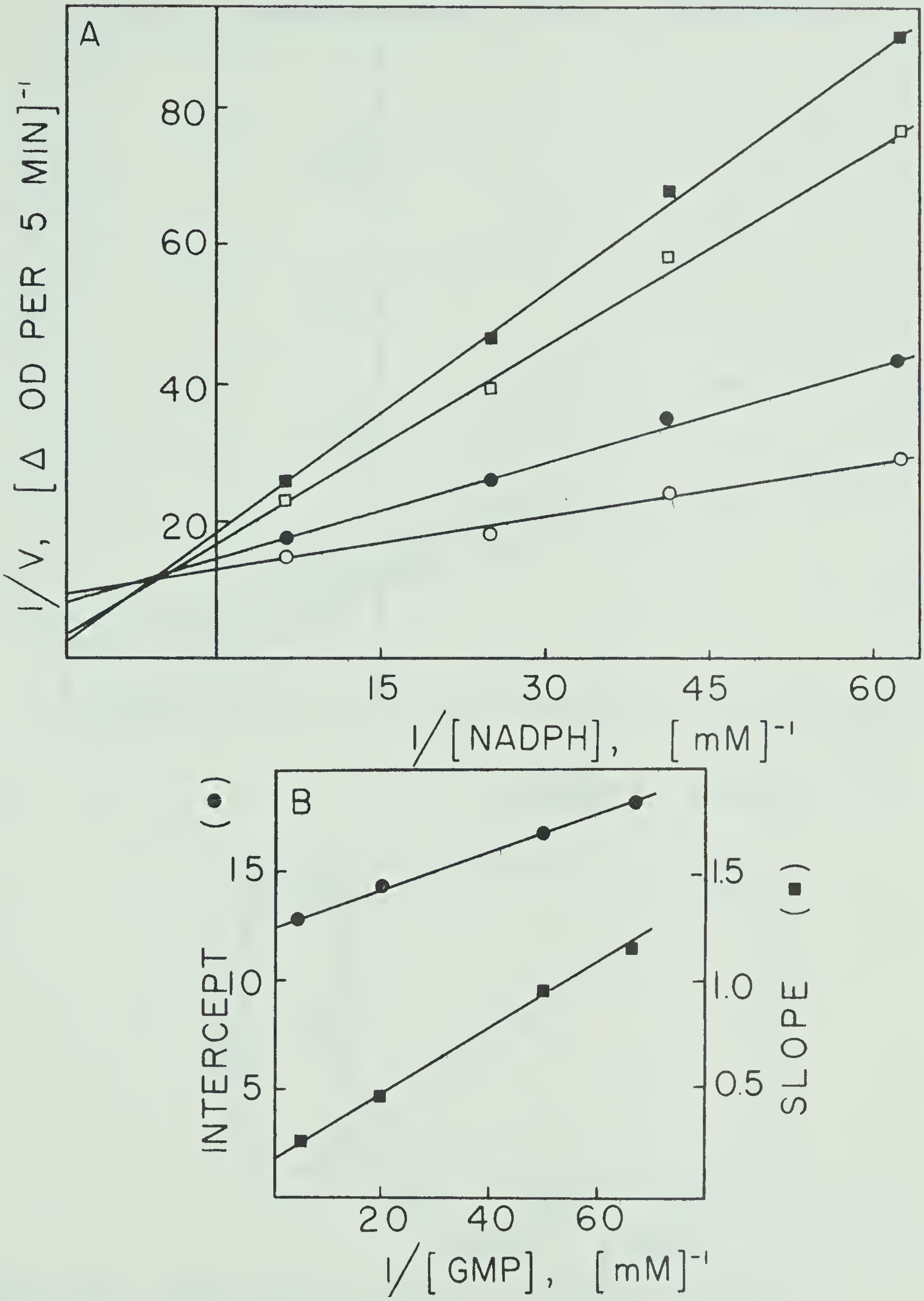


Figure 36: Initial velocity data for GMP reductase. Frame A; NADPH varied at fixed GMP concentrations of 1.5 x 10^{-5} M (■), 2.0 x 10^{-5} M (□), 5.0 x 10^{-5} M (●), and 2.0 x 10^{-4} M (○). Frame B; slopes and intercepts replotted against the reciprocal of the GMP concentration. OD changes measured at 340 mμ.

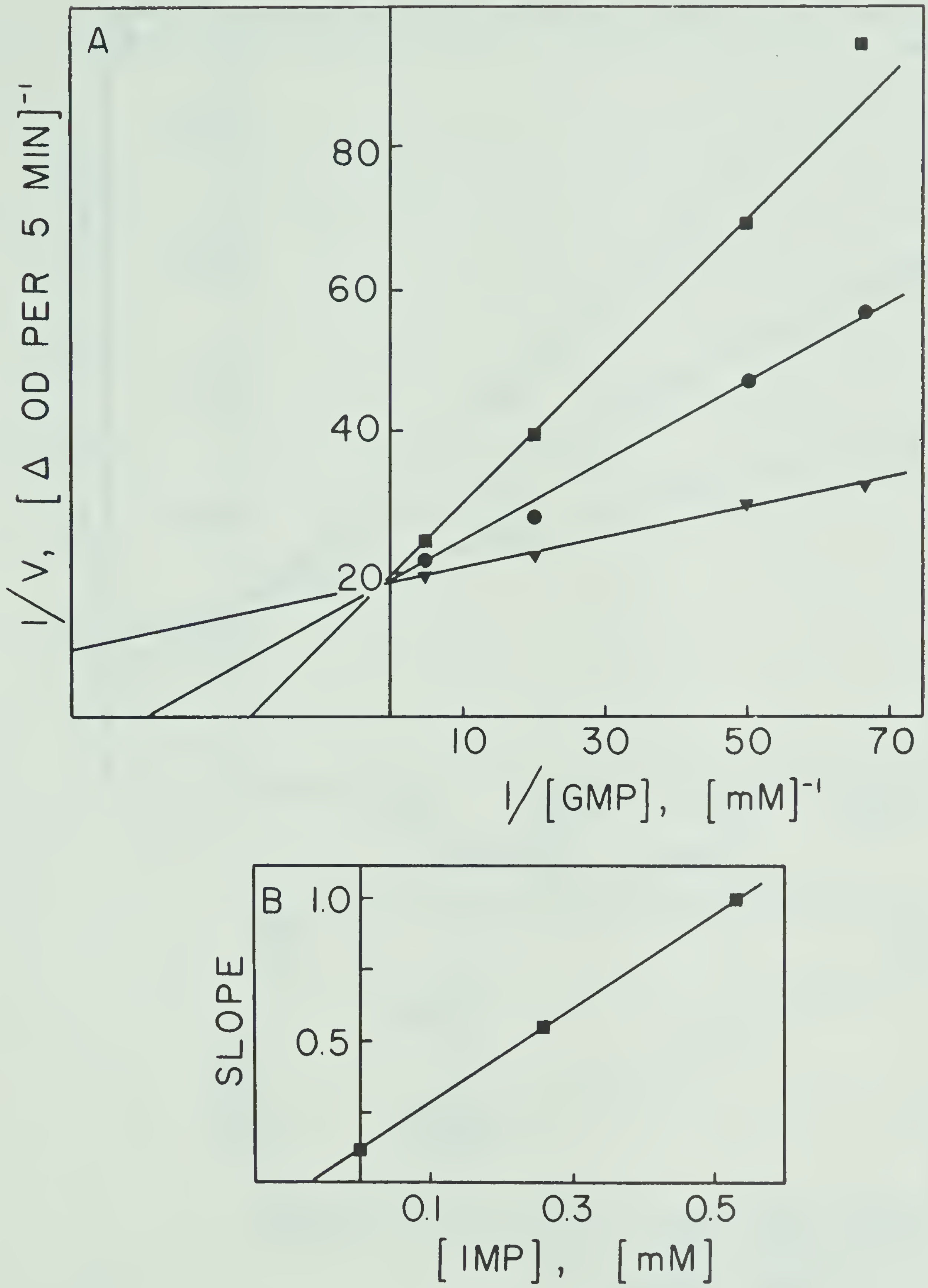


Figure 37: IMP product inhibion of GMP reductase. Frame A; GMP varied at IMP concentrations of 0 (▼), 2.6×10^{-4} M (●), and 5.3×10^{-4} M (■). NADPH concentration was 2.1×10^{-4} M. Frame B; replot of slopes against the IMP concentration. OD changes measured at 340 mμ.

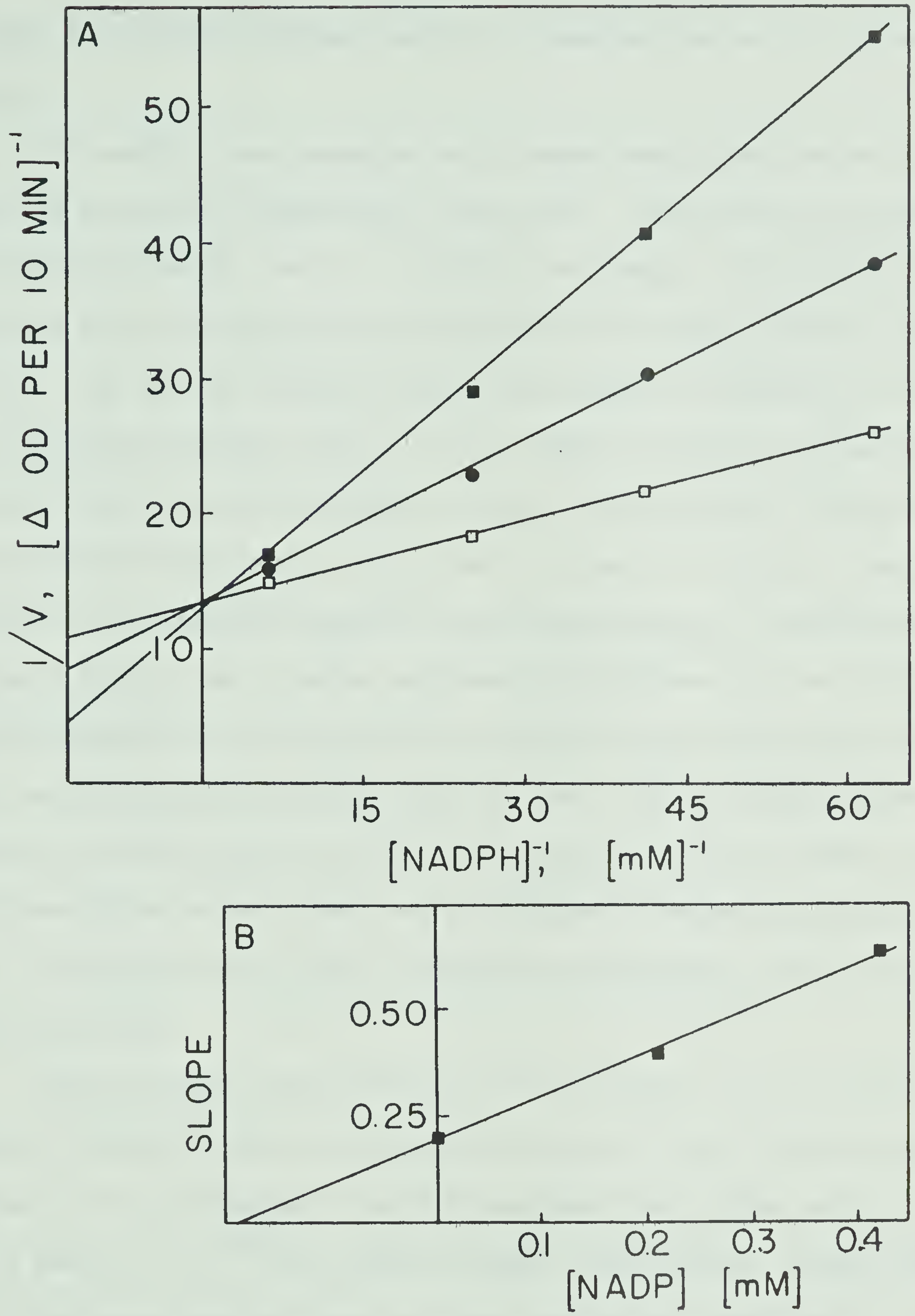


Figure 38: NADP product inhibition of GMP reductase. Frame A; NADPH varied at NADP concentrations of 0 (□), 2.2 x 10^{-5} M (●), and 4.4 x 10^{-4} M (■). GMP concentration was 2.0 x 10^{-4} M. Frame B; slopes replotted against the NADP concentration. OD changes measured at 340 mμ.

was made to determine whether ammonia or ammonium ion was the third product.

The complete rate equation for a random rapid-equilibrium Bi Ter model may be derived algebraically (Table IX). The underlying assumption in this derivation is that all substrate and product association or dissociation steps are very fast as compared to the actual reductive deamination. The initial velocity equation may again be obtained by setting product containing terms equal to zero. Table IX shows the slope and intercept functions for each substrate when this equation is placed in the reciprocal form, and it also gives the slope and intercept functions for the initial velocity equations containing products. The Michaelis constants (K_a,K_b) for a random rapid-equilibrium model are the dissociation constants for the particular substrate from the central complex, while the inhibition constants (K_{ia}, K_{iq}, etc.) are the dissociation constants for the particular substrate or product and free enzyme. These two dissociation constants for a given substrate are not necessarily equal, as binding of the first substrate may affect the affinity for the second substrate.

Table IX shows that replotting the intercepts of the initial-velocity reciprocal plots as in Figures 35 and 36 allows the Michaelis constants to be determined. For GMP reductase these values are 7.2×10^{-6} M and 13.3×10^{-6} M for GMP and NADPH, respectively. These values are of the order expected from our crude preliminary experiments. A Michaelis constant for GMP of 9.6×10^{-5} has been reported by Magasanik for this bacterial enzyme although no plots or experimental conditions were presented (21). The K_{ia} and K_{ib} inhibition constants may be calculated from the slopes of Figures 35 and 36 as seen in Table IX. Respect-

Table IX: Slope and Intercept Expressions for the Random Bi Ter Rate Equation.[a]

Substrate	Product	Slope (m)	Intercept (n)
GMP(A)	-	$\frac{K_aB + K_{ia}K_b}{V_1B}$	$\frac{1}{V_1} + \frac{K_b}{V_1B}$
NADPH(B)	-	$\frac{K_bA + K_{ia}K_b}{V_1A}$	$\frac{1}{V_1} + \frac{K_a}{V_1A}$
GMP(A)	IMP(P)	$\frac{K_bA + K_{ia}K_b}{V_1A}\left[1 + \frac{PK_{ia}K_b}{K_{ip}(K_aB + K_{ia}K_b)}\right]$	$\frac{1}{V_1} + \frac{K_b}{V_1B}$
NADPH(B)	NADP(Q)	$\frac{K_bA + K_{ia}K_b}{V_1A}\left[1 + \frac{QK_{ia}K_b}{K_{iq}(K_aB + K_{ia}K_b)}\right]$	$\frac{1}{V_1} + \frac{K_b}{V_1B}$

[a]

$$\frac{1}{v} = \frac{K_{ia}K_b}{ABV_1} + \frac{K_a}{V_1A} + \frac{K_b}{V_1B} + \frac{1}{V_1} + \frac{K_{ia}K_bR}{V_1K_{ir}AB} + \frac{K_{ia}K_bQ}{V_1K_{iq}AB} +$$

$$\frac{K_{ia}K_bP}{V_1K_{ip}AB} + \frac{K_{ia}K_bRQ}{V_1K'_{iq}K_{ir}AB} + \frac{K_{ia}K_bRP}{V_1K'_{ip}K_{ir}AB} + \frac{K_{ia}K_bPQ}{V_1K''_{iq}K_{ip}AB} +$$

$$\frac{K_{ia}K_bPQR}{V_1K''_{ip}K'_{iq}K_{ir}AB}$$

where K''_{iq} , K'_{iq} , and K_{iq} would represent dissociation constants of Q from the enzyme forms EPQ, ERQ, and EQ, respectively.

ive values of 8.8×10^{-5} and 16.5×10^{-5} M for GMP and NADPH are obtained. Figure 39-A shows the forward half of this random Bi Ter model with the corresponding substrate dissociation constants. It is seen that the initial binding of either substrate increases the affinity for the second substrate by a factor of twelve. How or why an enzyme enacts this type of phenomenon is not understood. Using the slope replots of Figures 37 and 38 it is also possible to calculate values of 3.0×10^{-5} M and 4.2×10^{-5} M for the dissociation constants of IMP and NADP with free enzyme. These values are subject to accumulative errors as other experimentally determined kinetic parameters are involved in the calculation (Table IX). These values are lower than those for the corresponding substrates and show that the initial binding of products is not in anyway contributing to the apparent irreversibility of this system.

The kinetic results show GMP may bind to GMP reductase in the absence of added NADPH. This is consistent with the postulate of the last chapter that the synthetic purine nucleotide inactivators are acting at the GMP binding site, as their effect similarly occurred in the absence of NADPH. The kinetic data suggests an interesting experiment that, unfortunately, has not been carried out. It was reported in the last chapter that 6×10^{-4} M NADPH had no protective effect over the inactivation of 6-chloropurine nucleotide. One might expect in the light of the kinetic evidence that saturating concentrations of NADPH may in fact promote inactivation since the affinity for this inhibitor may be increased by prior binding of NADPH as was the case for GMP. As stated earlier, the reason higher concentrations of NADPH were not used was that the correspondingly high optical densities could not be blanked out by the spectrophotometer.

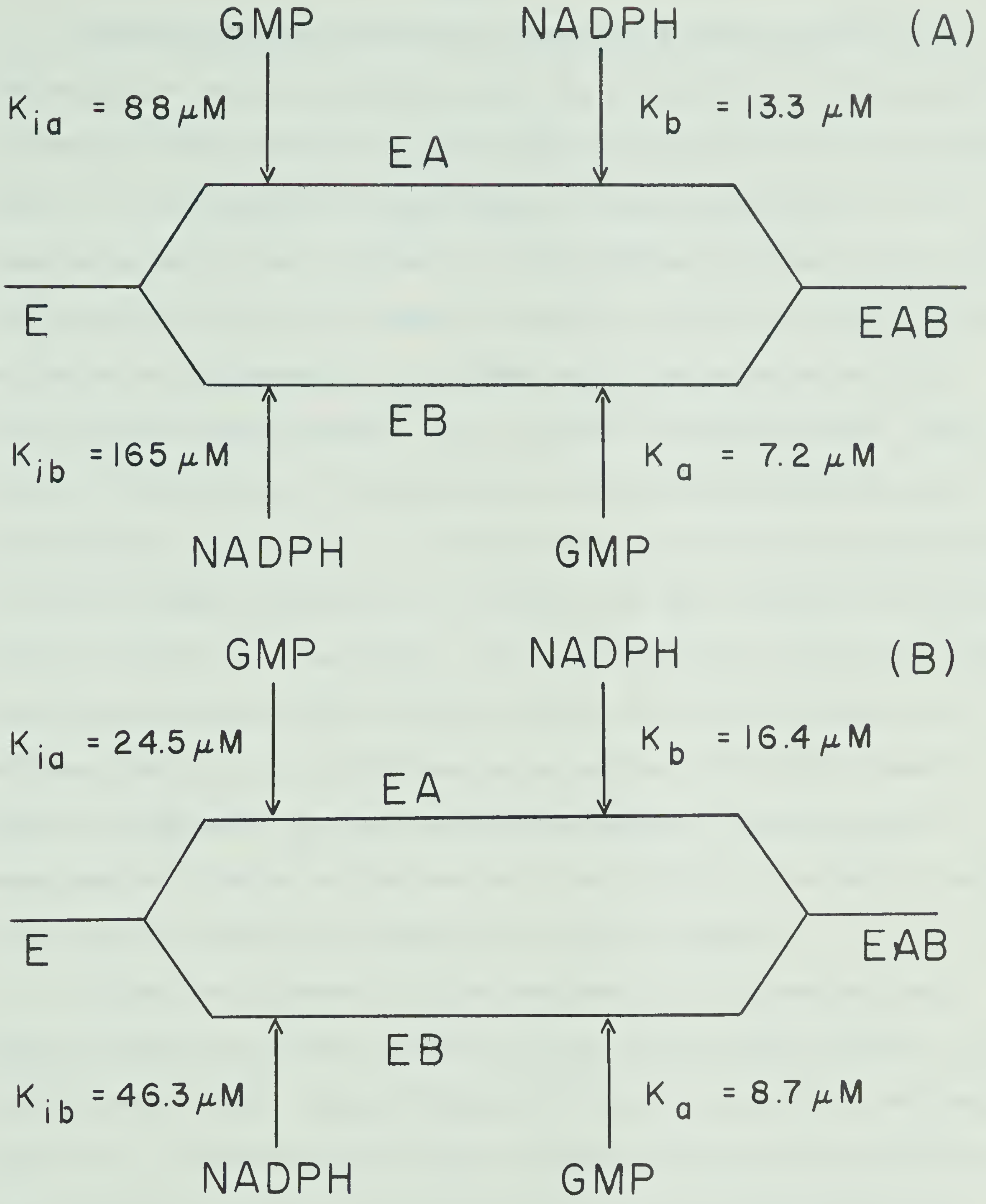

GMP
NADPH
(A)
K_{ia} = 88 μM
K_b = 13.3 μM
EA
E
EAB
EB
K_{ib} = 165 μM
K_a = 7.2 μM
NADPH
GMP
GMP
NADPH
(B)
K_{ia} = 24.5 μM
K_b = 16.4 μM
EA
E
EAB
EB
K_{ib} = 46.3 μM
K_a = 8.7 μM
NADPH
GMP

Figure 39: Substrate addition half of the proposed random Bi Ter kinetic model for GMP reductase showing substrate Michaelis and dissociation constants. Frame A; values obtained in absence of magnesium. Frame B; values obtained in presence of magnesium.

Figure 32 of the last chapter shows that a low concentration of GMP, while not offering protection from the 6-chloropurine nucleotide inactivation alone, did however afford protection under reaction conditions, that is, in the presence of added NADPH. A likely explanation for this observation may be that although 5 x 10^{-5} M GMP would saturate less than 50% of the GMP sites in the absence of NADPH it would give almost complete saturation in the presence of the NADPH due to the increased affinity resulting after NADPH binding. With increased saturation by GMP, there would be less binding of 6-chloropurine nucleotide and therefore less of the subsequent inactivation. A possible weakness in such an explanation is that the NADPH binding may also increase the GMP reductase's affinity for the 6-chloropurine nucleotide. One may rationalize around this problem by assuming that NADPH binding increases the relative affinity for GMP more than that for 6-chloropurine nucleotide. The kinetic results appear to complement the inactivation experiments in suggesting that these synthetic purine nucleotides are first binding at the GMP site and then reacting covalently to produce the inactive enzyme.

It has been reported (21) and confirmed in our laboratory that ATP is a strong reversible inhibitor of the bacterial GMP reductase. In neither case, though, had any divalent cation been included in the assay. Since it is the magnesium-ATP complex which is the active form of ATP in many biological systems, it was of interest to see if added magnesium affected this inhibition. During preliminary experiments, it was unexpectedly found that magnesium stimulated GMP reductase. About 1 x 10^{-3} M magnesium chloride was the lowest concentration giving the maximal stimulation. It was not the chloride ion which was stimulatory because 5 x 10^{-3} M sodium chloride was without effect. We had not studied cation

[illegible]

[illegible]

requirements since it had been reported that no monovalent or divalent cation requirement could be demonstrated for the bacterial GMP reductase (21). EDTA at 1.0×10^{-2} M had no effect on our reductase preparations which had suggested to us that no divalent cation was required.

The presence of 1×10^{-3} M magnesium chloride did not appear to alter the kinetic mechanism. Reciprocal plots were still linear and IMP and NADP were linear competitive inhibitors of GMP and NADPH, respectively. The Michaelis constants of 8.7×10^{-6} M for GMP and 16.4×10^{-6} M for NADPH did not seem to be significantly altered by the magnesium. However, with the magnesium chloride, the dissociation constant for GMP and free enzyme has dropped to 24.5×10^{-6} M and that for NADPH is reduced to 46.3×10^{-6} M. Both values have decreased by a factor of approximately 3.5. These results are shown in Figure 39-B. The dissociation constants of IMP and NADP with the free enzyme are reduced again by a factor of about 3 to 10.2×10^{-6} M and 14.0×10^{-6} M, respectively. The magnesium also gave rise to a 40% increase in the maximum velocity.

No satisfactory explanation of this magnesium effect is immediately apparent. Since the dissociation constants from the central complex, *i.e.* the Michaelis constants, are essentially unchanged, it would appear that any possible effect of magnesium on either substrate may be eliminated. As all the altered dissociation constants decrease by a factor of three to three and a half, it would seen that the role of magnesium is mediated on the enzyme itself. Whatever this role may be is unknown, but it most likely involves the same factors that confer on GMP reductase the increasing affinity phenomenon exhibited after the initial substrate binding. One assumes this to involve protein conformational changes but why and how such changes arise is still unknown.

IX. CONCLUSION

One of the goals of biochemistry and molecular biology is the elucidation of how an intact cell performs its many operations. It is therefore necessary to make some attempt to extrapolate *in vitro* data back to the *in vivo* situation. The problems incurred in making this *in vitro* to *in vivo* extrapolation are many, but they will only be solved by more intense investigation. As an example, the protein concentration, if indeed concentration is a meaningful term *in vivo*, is much higher in the intact cell than usually used in the *in vitro* enzyme studies. It is only very recently that interest has developed in studying enzyme kinetics with enzyme concentrations in the order of milligrams per ml instead of the usual micrograms per ml or less (95). When the concentration of enzyme and substrates become of the same order of magnitude as is probably the case in many *in vivo* situations, the present day kinetic theories will likely no longer be applicable, since in these cases the steady-state assumption may no longer be valid. Similarly, the intracellular pH and metabolite concentrations are as yet relatively uncharted variables. The extent of compartmentization with its possible local pH and metabolite gradients is unknown although this phenomenon has been called upon numerous times in the literature to explain various experimental results. Problems of this nature are undoubtedly complicated as evidenced by the lack of available information, but information of this type is necessary before the *in vitro* to *in vivo* extrapolation can be readily

made. Similarly, only when the intracellular environment is more documented will it be really feasible to study cellular control mechanisms.

The *in vitro* data on a given enzyme may be examined to see if it it is consistent with the known cellular conditions, and it will then serve as a base line if *in vivo* studies are undertaken. The Michaelis constant for IMP with the IMP dehydrogenases so far studied is relatively low and of the order of 2 x 10^{-5} M. Such a value would seemingly be required for significant rates of XMP formation, as the intracellular IMP concentration is also believed to be very low. The intracellular level of IMP is lower than that of AMP which has been estimated at 1-3 x 10^{-5} M in *E. coli* (96) and in human red blood cells (97).

The level of cellular NAD will also affect the IMP to XMP conversion rate. No data was found on the bacterial levels of this coenzyme, but in the rat, liver has the highest content of NAD at about 1.2 x 10^{-3} M (98). It also appears that 30-40% of the cellular NAD is in the reduced form. Therefore, an IMP dehydrogenase with a Michaelis constant for NAD of 1 x 10^{-3} M will not be operating at full capacity. It must always be kept in mind, though, that estimates of the cellular concentration of a metabolite are based on the amount of metabolite and the cell volume, and they do not contain any consideration for higher local concentrations which may arise through compartmentization. All things being equal, it appears that the Sarcoma 180 IMP dehydrogenase would be more efficient than the bacterial enzyme as its Michaelis constant for NAD is lower at 8 x 10^{-5} M (19). It is not, however, known whether this difference represents a difference in enzyme properties or is merely the result of purification procedures. The intracellular pH will also affect the NAD affinity as the Michaelis constant

for NAD drops from 1×10^{-3} M at pH 8.1 to 0.2×10^{-3} M at pH 6.0 for the bacterial enzyme. The cellular Michaelis constant for NAD may then be lower than 1×10^{-3} M as the intracellular pH is probably in the range of 7 to 7.5.

The problems encountered with an *in vitro* to *in vivo* extrapolation make the means of the cellular control of IMP dehydrogenase unclear, but certain possibilities are apparent. First of all, if it is assumed that the cellular concentrations of IMP and NAD approximate their respective K_M values, it is seen that slight fluctuations in substrate levels could drastically change the rate of the IMP to XMP conversion. One factor which may cause the level of a particular substrate to vary is the presence of other enzymes which also utilize this substrate. IMP is also a substrate for adenylosuccinate synthetase which is the enzyme catalyzing the first step of the two-step process leading from IMP to AMP (8). Since no complete kinetic analysis has been reported on this enzyme from any source, it is not possible to even compare these two enzymes at the *in vitro* level. However, an apparent Michaelis constant of 5.4×10^{-5} M for IMP and the synthetase from *E. coli* has been reported (99), and this value implies that both the adenylosuccinate synthetase and the IMP dehydrogenase may have equal affinities for IMP. The relative amount of IMP channelled into either adenine or guanine nucleotides will then depend on the relative concentrations of IMP dehydrogenase and adenylosuccinate synthetase together with the availability of all the involved substrates and cofactors. Similarly, there are numerous dehydrogenases which use NAD as the pyridine coenzyme, and the utilization of the cellular NAD by these enzymes will have an influence on the level of NAD available to the IMP dehydrogenase. Any large fluctuations in intracellular metabol-

ite concentrations are presumably of a rather limited duration, but they could still play a role in regulating enzymic activity.

Mager and Magasanik reported in their early studies on the bacterial IMP dehydrogenase that GMP inhibited this system (21). They postulated that GMP may then be able to regulate its own synthesis through a feedback type of control. The subsequent reports from our laboratory on the bacterial enzyme (20) and from Sartorelli's laboratory on the Sarcoma 180 enzyme (19) have demonstrated GMP to be a competitive inhibitor with respect to IMP. The reported dissociation constants were 1.3 and 3.0 x 10^{-4} M, respectively. Whether GMP can serve as an effective control over its own synthesis must still be considered unresolved until more is known about cellular fluctuations in nucleotide pools. The normal intracellular concentration of GMP is probably slightly higher than IMP but still lower than AMP (99). Since it is unlikely that intracellular concentrations of IMP and NAD are sufficient to saturate the dehydrogenase, this system would be expected to be very sensitive to competitive-type inhibitors, and hence GMP may indeed have some influence over its own synthesis. Whether GMP serves as an intracellular control or not, it would be expected to bind at the IMP site since it is structurally similar to IMP and XMP, and it is also a competitive inhibitor of IMP.

A recent communication claimed to have showed that the binding site on IMP dehydrogenase for GMP and IMP are distinct (100). The evidence was based on two points. Firstly, a pH vs. pK_{IMP} plot and a pH vs. pK_{iGMP} plot gave different profiles. These workers argued that if one site was involved similar profiles should be expected. This sounds well in theory, but it is not possible to compare the Michaelis constant for

IMP with a dissociation constant for an inhibitor. As has been shown in Chapter IV, the Michaelis constant for IMP does not equal the dissociation constant at all pH values. Furthermore, all of the K_{IMP} and K_{iGMP} values reported in this study were obtained at only one NAD or GMP concentration, and hence can not be compared at different pH values as they are only apparent constants. It is also possible that the pH vs. the negative logarithm of the dissociation constant plot for IMP and GMP might differ even if both were reacting at the same site. This might be expected if the C-6 oxygen of IMP or the C-6 amino and C-2 oxygen of GMP influenced binding, as all these groups have different pK values.

The second line of evidence for a GMP control site was the appearance of nonlinear reciprocal plots when GMP was present (100). These reported variations from linearity were not substantiated by sufficient experimental points or by statistical curve fitting with the proper weighting factors (101). This forementioned paper, in the opinion of this writer, contributes nothing to the question of whether or not IMP dehydrogenase has a specific site for GMP binding, and is just one example of the excessive and unobjective interest displayed by many workers in allosteric enzymes.

The role GMP reductase plays in channelling guanine to adenine nucleotides via IMP has not been studied to any extent. Due to the specific requirement for NADPH it may be argued that this enzymatic step is of an anabolic nature as opposed to a means of routinely adjusting extracellular nucleotide pool sizes. It has been proposed that a second pyridine coenzyme, such as NADPH, which is not as easily oxidized by molecular oxygen as is NADH is required as a storehouse of molecular hydrogen which is consistently available for the biosynthesis of proteins,

lipids and nucleic acids (102). This idea is in accord with the fact that usually in excess of 90% of the total cellular triphosphopyridine nucleotide is in the reduced form while only 30-40% of the diphosphopyridine nucleotide is in the reduced state. The GMP reductase system may then serve mainly in the synthesis of adenine nucleotides when a source of exogenous guanine is available. This would be of economic advantage from an energy viewpoint since the purine ring would no longer have to be synthesized by the purine _de novo_ pathway.

It has been shown in bacteria that exogenous adenine or guanine will support synthesis of both nucleic acid adenine or guanine (32, 33, 34). The purineless mutant strain PD-1 derived from _Aerobacter aerogenes_, strain 1033, for instance, can not synthesize purines _de novo_ and requires for growth either free purine bases or 4-amino-5-imidazolecarboxamide which is one of the later intermediates in the _de novo_ pathway (11). If then guanine can serve as the purine source, the GMP reductase activity must be adequate to give rise to a level of adenine nucleotides sufficient for growth. GMP reductase activity has been demonstrated from only a few mammalian sources, two of which are bone-marrow (35) and erythrocytes (39). Bone-marrow has been reported to process a limited capacity for synthesizing purine nucleotides and to rely in part on preformed liver purines (103). The mature erythrocyte has completely lost the ability to synthesize purine nucleotides _de novo_ (104). It would be interesting to compare the levels of GMP reductase in the forementioned cells with those having an adequate purine _de novo_ pathway. Similarly, it would be of interest to resolve whether this enzyme is present in liver (37, 38), and if so, to determine its function.

As mentioned earlier the intracellular concentration of GMP is probably less than 3 x 10^{-5} M. The low Michaelis constant for GMP exhibited by the GMP reductase would be required for substantial rates of the deamination of GMP. The cellular concentration of NADPH is likely not to be in excess of 5 x 10^{-5} M (98). For the level of NADPH in human erythrocytes, a value of 0.04 - 0.05 μmoles of phosphorus per ml of packed cells has been reported (97). Hence, even in the presence of magnesium, it is unlikely that the GMP reductase could operate much in excess of 50% of its maximal velocity. As with the IMP dehydrogenase, such an unsaturated system would be sensitive to many factors. When cells are growing and actively synthesizing nucleic acids, an increased supply of ribose will be required. This ribose arises from the glucose oxidative pathway which also makes available a supply of NADPH. This coenzyme is then presumably available to the reductase if guanine is serving as the main source of nucleic acid purines.

Mager and Magasanik had reported and we have confirmed the observation that ATP is a powerful inhibitor of the bacterial GMP reductase (21, 84). The triphosphate appears more potent in inhibiting this system than any other natural purine nucleotide. In the preliminary experiments we have done to date, it appears that ATP is competitive with IMP. No data is available for the ATP inhibition with respect to NADPH. The lack of protection offered by ATP to the GMP reductase against the inactivation by 6-chloropurine nucleotide suggested a separate site may exist for this triphosphate. If ATP were binding at the coenzyme site it is difficult to understand why GMP served to completely reverse the ATP inhibition, whereas NADPH did not seem to have an effect. This could conceivably result if ATP bound very strongly to

free enzyme but hardly at all to the enzyme-GMP complex. Neither of the studies on the ATP inhibition of GMP reductase (21, 84) included magnesium or any other divalent cation in the assay mixture. In the light of our recent observations on the magnesium stimulation of GMP reductase it would be of interest as to if magnesium had any effect on the ATP inhibition. We have some limited evidence from very recent experiments that the ATP inhibition may be decreased by the presence of magnesium. More work is required to clarify this point.

It is not easy to interpret this in vitro ATP inhibition in terms of a possible in vivo control on the GMP reductase. Data on E. coli (96) and S. typhimurium (105) indicate the intracellular level of ATP may be about 1×10^{-3} M. This suggests that GMP reductase would be essentially inoperative if the estimated cellular levels of GMP and ATP were those confronting the enzyme. The cellular levels of ATP in E. coli and S. typhimurium appear to be rather independent of the growth rate (96, 105), whereas, it has been shown in A. aerogenes that ATP levels varied over a six- to eight-fold range when the growth rate was varied by means of the carbon and energy supply (106). The P-14 mutant of A. aerogenes used in our studies will have a slower growth rate since it is being grown on limiting guanine, and it will presumably have a lower level of ATP. Perhaps this reduction in ATP concentration would be sufficient to allow the reductase to become operative. One is still faced with the problem of how wild type E. coli and S. typhimurium can convert guanine to adenine nucleotides in the presence of a level of ATP which would be expected from the in vitro data to completely inhibit the GMP reductase. Problems of this nature will only be solved by careful in vivo studies and when more is known about the intracellular environment.

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